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JCS83 U.S. PTO

PATENT  
Attorney Docket No. AURO1130-2

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JCS83 U.S. PTO  
09/468002  
12/20/99

Sir:

Transmitted herewith for filing is the **continuation** patent application of

Inventor(s): Paul Negulescu; Stefan Offermanns; Melvin Simon; Charles Zuker

For: **PROMISCUOUS G-PROTEIN COMPOSITIONS AND THEIR USE**

This is a request for filing a **continuation** application under 37 C.F.R. 1.53(b), of prior U.S. Application Serial No. 08/878,801; filed on June 19, 1997, issuing on December 21, 1999 as U.S. Patent No. 6,004,808, which claims priority under 119(e)(1) from U.S. Provisional Patent Application Serial No. 60/020,234, filed June 21, 1996.

Enclosed are:

	<u># of Pages</u>
Specification	53
Claims	6
Abstract	1
Drawings [# of Sheets]	8
Combined Declaration and Power of Attorney	5
Permission to Use Sequence Listing and Paper Copy	2
Preliminary Amendment	2
Information Disclosure Statements	2
PTO 1449	4

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Filing fee to be charged to Deposit Account No. 07-1895 in amount of \$760.00

This is a request for filing a **continuation** application under 37 C.F.R. 1.53(b), of prior U.S. Application Serial No. 08/878,801; filed on June 19, 1997, issuing on December 21, 1999 as U.S. Patent No. 6,004,808, which claims priority under 119(e)(1) from U.S. Provisional Patent Application Serial No. 60/020,234, filed June 21, 1996, the contents of which is incorporated by reference in its entirety herein.

In re of Application of  
Negulescu et al.  
Filed: Herewith  
Page 2

PATENT  
Attorney Docket No.: AURO1130-2

No payment of the issue fee, abandonment of, or termination of proceeding has occurred in the above-identified prior application.

1. ☐ Cancel in this application original claim(s) \_\_\_\_\_.
2. ☒ A preliminary amendment is enclosed.

The filing fee has been calculated as shown below:

For	Number Filed		Number Extra		Rate		Fee	
					Small Entity	Other Entity	Small Entity	Other Entity
Total Claims	1	=	20	X	\$9	\$18	= \$0.00	\$ 0.00
Independent Claims	1	=	1	X	\$39	\$78	= \$0.00	0
Multiple Dependent Claims Presented: <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No					\$130	\$260		0
BASIC FEE					\$380	\$760	\$0.00	\$ 760.00
TOTAL FEE							\$0.00	\$760.00

3. ☒ Please charge my Deposit Account No. 07-1895 the TOTAL FEE of \$760.00 which covers the filing fee for this application. A duplicate copy of this sheet is enclosed.
4. ☒ The Assistant Commissioner is hereby authorized to charge payment of the following fees associated with this communication or credit any overpayment to Deposit Account No. 07-1895. A duplicate copy of this sheet is enclosed.  
☒ Any additional filing fees required under 37 C.F.R. 1.16.  
☒ Any patent application processing fees under 37 C.F.R. 1.17.
5. ☒ Amend the specification by inserting after the title on page 1:

This is a request for filing a **continuation** application under 37 C.F.R. 1.53(b), of prior U.S. Application Serial No. 08/878,801; filed on June 19, 1997, issuing on December 21, 1999 as U.S. Patent No. 6,004,808, the entire contents of which is [are] hereby incorporated by reference herein, which claims priority under 119(e)(1) from U.S. Provisional Patent Application Serial No. 60/020,234, filed June 21, 1996.

6. ☐ A verified statement claiming small entity status was filed in parent application, Serial No. \_\_\_\_\_, filed on \_\_\_\_\_, and such status is still proper.

In re of Application of  
Negulescu et al.  
Filed: Herewith  
Page 3

7. X The prior application is assigned of record to Aurora Biosciences Corporation, on Reel 8889, Frame 0595 on January 12, 1998.
8. X The power of attorney in the prior application is to Lisa A. Haile, Registration No. 38,347.
9. X Please transfer the drawings from the prior application to the new application.
10. X A copy of the prior application as filed is enclosed, including a copy of a Combined Declaration and Power of Attorney filed in parent application, U.S. Application Serial No. 08/878,801, filed on June 19, 1997.
11. X Permission to Use Sequence Listing of parent priority is enclosed along with paper copy of Sequence Listing.
12. X Information Disclosure Statements filed in the prior application under 37 C.F.R. 1.97 are hereby made of record. Copy of PTO-1449 is enclosed.

Address all future communications to:

Lisa A. Haile, Ph.D.  
GRAY CARY WARE & FREIDENRICH LLP  
4365 Executive Drive, Suite 1600  
San Diego, California 92121-2189  
Telephone: 858-677-1456  
Facsimile: 858-677-1465

In re of Application of  
Negulescu et al.  
Filed: Herewith  
Page 4

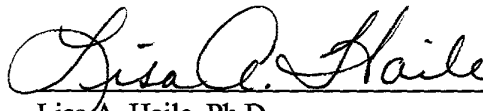
PATENT  
Attorney Docket No.: AURO1130-2

The undersigned states that the enclosed application papers comprise a true copy of the prior application as filed.

Respectfully submitted,

Date:

12/20/99



Lisa A. Haile, Ph.D.  
Attorney for Applicant  
Registration No. 38,347  
Telephone: 858-677-1456  
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GRAY CARY WARE & FREIDENRICH LLP  
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Negulescu et al.

Parent Application No.: 08/878,801

Parent Filed: June 19, 1997

Application No.: Unassigned

Filed: Herewith

For: AMIDASE

) Group Art Unit: Unassigned  
)

) Examiner: Unassigned  
)

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) Michael Bayley  
) (TYPED OR PRINTED NAME OF PERSON MAILING PAPER OR FEE)

) Michael Bayley  
) (SIGNATURE OF PERSON MAILING PAPER OR FEE)

Assistant Commissioner for Patents  
Washington, D.C. 20231

**PRELIMINARY AMENDMENT**

Sir:

Prior to examination of the above-identified new patent application, please consider the following amendments and remarks:

In re Application of:  
Negulescu et al.  
Filed: Herewith  
Page 2

PATENT  
Attorney Docket No.: AURO1130-2

**IN THE CLAIMS:**

Please delete claims 2-62.

**REMARKS**

No new matter has been added. Applicant respectfully requests entry of this amendment.

If the Examiner would like to discuss any of the issues raised in the Office Action, Applicant's representative can be reached at (858) 677-1456. While it is not believed that any fees are due, if the PTO finds any additional fees due, or make any credits, to Deposit Account No. 07-1895.

Respectfully submitted,

Dated: 12/30/99



Lisa A. Haile, Ph.D.  
Registration No. 38,347  
Telephone: 858-677-1456  
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GRAY CARY WARE & FREIDENRICH LLP  
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San Diego, CA 92121-2189

**PROMISCUOUS G-PROTEIN COMPOSITIONS AND THEIR USE****CROSS REFERENCE TO RELATED APPLICATIONS**

This application claims priority under 35 U.S.C. Section 119 to provisional patent application 60/020,234 filed on June 21, 1996, by Negulescu et al., which is herein incorporated by reference and of which this application is a continuation in part.

**FIELD OF THE INVENTION**

The invention relates to compositions and methods for identifying G-protein coupled receptors (GPCRs) and compounds that modulate activity of G-proteins or their receptors.

**BACKGROUND**

Many physiological signals (e.g., sensory, hormonal and neurotransmitter signals) are transduced from extracellular to intracellular environments by cell surface receptors termed G-protein coupled receptors (GPCRs) (for a review, see Neer, 1995, Cell 80:249-257). Typically, GPCRs contain seven transmembrane domains. Putative GPCRs can be identified on the basis of sequence homology to known GPCRs.

GPCRs mediate signal transduction across a cell membrane upon the binding of a ligand to an extracellular portion of a GPCR. The intracellular portion of a GPCR interacts with a G-protein to modulate signal transduction from outside to inside a cell. A GPCR is therefore said to be "coupled" to a G-protein. G-proteins are composed of three polypeptide subunits: an  $\alpha$  subunit, which binds and hydrolyzes GTP, and a dimeric  $\beta\gamma$  subunit. In the basal, inactive state, the G-protein exists as a heterotrimer of the  $\alpha$  and  $\beta\gamma$  subunits. When the G-protein is inactive, guanosine diphosphate (GDP) is associated with the  $\alpha$  subunit of the G-protein. When a GPCR is bound and activated by a ligand, the GPCR binds to the G-protein heterotrimer and decreases the affinity of the  $G\alpha$  subunit for GDP. In its active state, the G subunit exchanges GDP for guanine

triphosphate (GTP) and active  $G\alpha$  subunit disassociates from both the receptor and the dimeric  $\beta\gamma$  subunit. The disassociated, active  $G\alpha$  subunit transduces signals to effectors that are “downstream” in the G-protein signalling pathway within the cell. Eventually, the G-protein’s endogenous GTPase activity returns active G subunit to its inactive state, in which it is associated with GDP and the dimeric  $\beta\gamma$  subunit.

Numerous members of the heterotrimeric G-protein family have been cloned, including more than 20 genes encoding various  $G\alpha$  subunits. The various G subunits have been categorized into four families, on the basis of amino acid sequences and functional homology. These four families are termed  $G\alpha_s$ ,  $G\alpha_i$ ,  $G\alpha_q$ , and  $G\alpha_{12}$ . Functionally, these four families differ with respect to the intracellular signaling pathways that they activate and the GPCR to which they couple.

For example, certain GPCRs normally couple with  $G\alpha_s$  and, through  $G\alpha_s$ , these GPCRs stimulate adenylyl cyclase activity. Other GPCRs normally couple with  $G\alpha_q$ , and through  $G\alpha_q$ , these GPCRs can activate phospholipase C (PLC), such as the  $\beta$  isoform of phospholipase C (PLC $\beta$ ) (Sternweis and Smrcka, 1992, Trends in Biochem. Sci. 17:502-506).

Certain G-proteins are considered “promiscuous” G-proteins because their G subunits allow them to couple with GPCRs that normally couple with G-proteins of other families. For example, two members of the  $G\alpha_q$  family, human  $G\alpha_{16}$  and its murine homolog  $G\alpha_{15}$ , have been shown in transient cell-based systems to possess promiscuous receptor coupling. Although G-proteins having these G subunits are promiscuous with respect to the GPCR with which they couple, these G-proteins retain the ability to couple with a specific downstream effector. In other words, regardless of which receptor is used to activate these G-proteins, the active promiscuous G subunit nonetheless activates PLC $\beta$ .



## SUMMARY OF THE INVENTION

The invention provides for the first time, a stable, isolated cell that expresses, from a construct, a  $G\alpha$  subunit of a promiscuous G-protein (e.g.,  $G\alpha_{15}$  or  $G\alpha_{16}$ ). In a preferred embodiment, a polynucleotide encoding a promiscuous G subunit is linked to an inducible promoter on the construct. To detect activation of the promiscuous G-protein, the cell can include an additional construct that includes a reporter gene operably linked to a promoter that is activated (usually indirectly) by an active G subunit of a promiscuous G-protein. For the first time, these cells allow occupation of any G-protein coupled receptor (GPCR) by a ligand to be detected using a signal transduction detection system, such as expression of a reporter gene. Other signal transduction detection systems include detecting changes in intracellular activity, such as methods of detecting G-protein activation from changes in calcium levels in the cell. Preferred methods for detecting expression of the reporter gene involve detecting a change in fluorescence emission from a sample that includes the cell containing the reporter gene.

Another key aspect of the invention is functional selection of stable cell lines. Stable cell lines can be functionally selected using a signal transduction detection system as described herein. Stable cells are generated that tolerate the expression of a target protein (such as an ion channel, kinase, phospholipase, phosphatase, transcription factors or GPCR) or a signal transduction coupling protein (e.g. G protein) or both.

The cells of the invention can be employed in methods for (i) determining whether a polypeptide is a GPCR for a given ligand; (ii) determining whether a “test” ligand is a ligand for a given GPCR; (iii) functionally characterizing the ability of a ligand to activate various GPCRs; and (iv) determining whether a compound modulates signal transduction in a cell (e.g., as an agonist or antagonist).

Another aspect of the invention includes, a method of identifying of a ligand for a GPCR, the method comprising:

- a) contacting a cell with a test chemical, wherein said cell is expressing a GPCR and arises from a cell line subjected to functional cell analysis with a signal transduction detection system ; and
- b) detecting a signal with a signal transduction detection system.

A related aspect of the invention includes, a method for identifying modulators of signal transduction in a cell, the method comprising:

- c) contacting a cell with a compound that directly or indirectly activates a  $G\alpha$  protein encoded by a polynucleotide, wherein said cell arises from a cell line subjected to functional cell analysis with a signal transduction detection system,
- d) contacting said cell with a test chemical, and
- e) detecting a signal with a signal transduction detection system.

The invention also includes, a method for identifying a GPCR for a given ligand or method of identifying a modulator of a GPCR, the method comprising:

- f) expressing a putative GPCR or a GPCR of known function in a cell, wherein said cell arises from a cell line subjected to functional cell analysis with a signal transduction detection system;
- g) contacting said cell with a test chemical or a ligand known to be a GPCR ligand; and
- h) detecting a calcium level within said cell.

Also included within the invention, are kits that components for signal transduction detection systems and cells of the invention.

The invention also includes methods of identifying modulators that do not employ a GPCR, but which employ direct activators of G-proteins.

### **Brief Description of the Drawings**

Fig. 1 shows uses of promiscuous G $\alpha$ -protein to detect activation of a variety of GPCRs. Three major classes of GPCRs are diagrammed coupling to their endogenous signaling cascade. Promiscuous G-protein expression will allow various classes to couple to the PLC cascade.

Fig. 2 shows one embodiment of the invention that can be used for screening for Gq type G-protein activation. Modulation of GPCR activity initiates signaling cascade via PLC. PLC signals can be detected using NFAT responsive element linked to a transcriptional readout (e.g. reporter gene).

Fig. 3 is a copy of a photograph of a Western blot. Lanes corresponding to samples that lacked or contained doxycyclin are indicated by “-” and “+” respectively. Lanes 1-4 show inducible expression of G $\alpha_{15}$  in two distinct clones of COS-7 cells. Lanes 5-8 show inducible expression of G $\alpha_{16}$  in two distinct clones. G $\alpha_{15}$  and G $\alpha_{16}$  each appear as a species having a molecular weight of approximately 43 kDa in each of the “+” lanes. As a negative control, COS-7 cells were analyzed in lane 9. The predicted molecular weight of G $\alpha$  subunit is 43-45 kDa.

Fig. 4A is a graph showing the ionomycin dose response, as measured by fluorescence emission of living cells that express  $\beta$ -lactamase reporter gene, and which were contacted with a fluorogenic  $\beta$ -lactamase substrate. Because the NFAT response element usually requires both a calcium increase and protein kinase C activation, these cells were also treated with 10nm PMA Fig. 4B is a graph showing the PMA dose response of living cells that express a  $\beta$ -lactamase reporter gene, and which were contacted with a fluorogenic  $\beta$ -lactamase substrate. In this case, all samples were also treated with 2 $\mu$ M ionomycin.

Fig. 5 is a graphic representation of the emission spectrum of the  $\beta$ -lactamase substrate CCF2 before and after it is cleaved by  $\beta$ -lactamase.

Fig. 6 shows the results of an NFAT  $\beta$ -lactamase transcription based assay using a heterologously express GPCR (Gq subtype) in the presence of agonist, agonist and antagonist or solvent for agonist (“non-stimulated” control).

Fig. 7 shows activation of a Gas subtype GPCR (panels A-C) and a Gai subtype GPCR (panels D-F) using promiscuous Gα protein in a cell-based (transient transfection of all constructs) calcium indicator assay (FURA-PE3).

Panel A: 60 seconds after starting of the experiment, 10μM agonist solution was added to the cells transfected by pCIS/Gα16 and Gs-receptor expression plasmids.

Panel B: 60 seconds after starting of the experiment, 10μM agonist solution was added to the cells transfected by both pCIS/Gα 16 alone.

Panel C: 60 seconds after starting of the experiment, 10μM agonist solution was added to the cells transfected by Gas receptor expression plasmid alone.

Panel D: 60 seconds after starting of the experiment, 10μM agonist solution was added to the cells transfected by pCIS/Gα16 and Gai-receptor expression plasmids.

Panel E: 60 seconds after starting of the experiment, 10μM agonist solution was added to the cells transfected by pCIS/Gα 16 alone.

Panel F: 60 seconds after starting of the experiment, 10μM agonist solution was added to the cells transfected by Gai-receptor expression plasmid alone.

Fig. 8 shows activation of a Gas subtype GPCR using promiscuous Gα protein in a cell-based (stably transfected constructs for both the promiscuous Gα-protein and GPCR) calcium indicator assay.

Panel A: Calcium imaging of the Gα15/Gas-receptor dual stable pool-2. 10μM agonist was added 40 seconds after the starting of the experiment.

Panel B: Calcium imaging of the Gas-receptor stable pool-2. 10μM agonist was added 40 seconds after the starting of the experiment.

Panel C: Calcium imaging of the Gα15 stable pool-H. 10μM agonist was added 40 seconds after the starting of the experiment.

## DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in spectroscopy, drug discovery, cell culture, and molecular genetics, described below are those well known and commonly employed in the art. Standard techniques are typically used for preparation of signal detection, recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, and lipofection). The techniques and procedures are generally performed according to conventional methods in the art and various general references (see generally, Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., and Lakowicz, J.R. *Principles of Fluorescence Spectroscopy*, New York: Plenum Press (1983) for fluorescence techniques, which are incorporated herein by reference) which are provided throughout this document. Standard techniques are used for chemical syntheses, chemical analyses, and biological assays. As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

"Fluorescent donor moiety" refers to the radical of a fluorogenic compound, which can absorb energy and is capable of transferring the energy to another fluorogenic molecule or part of a compound. Suitable donor fluorogenic molecules include, but are not limited to, coumarins and related dyes xanthene dyes such as fluoresceins, rhodols, and rhodamines, resorufins, cyanine dyes, bimanes, acridines, isoindoles, dansyl dyes, aminophthalic hydrazides such as luminol and isoluminol derivatives, aminophthalimides, aminonaphthalimides, aminobenzofurans, aminoquinolines, dicyanohydroquinones, and europium and terbium complexes and related compounds.

"Quencher" refers to a chromophoric molecule or part of a compound, which is capable of reducing the emission from a fluorescent donor when attached to the donor. Quenching may occur by any of several mechanisms including fluorescence resonance energy transfer, photoinduced electron transfer, paramagnetic enhancement of

intersystem crossing, Dexter exchange coupling, and excitation coupling such as the formation of dark complexes.

"Acceptor" refers to a quencher that operates via fluorescence resonance energy transfer. Many acceptors can re-emit the transferred energy as fluorescence. Examples include coumarins and related fluorophores, xanthenes such as fluoresceins, rhodols, and rhodamines, resorufins, cyanines, difluoroboradiazaindacenes, and phthalocyanines. Other chemical classes of acceptors generally do not re-emit the transferred energy. Examples include indigos, benzoquinones, anthraquinones, azo compounds, nitro compounds, indoanilines, di- and triphenylmethanes.

"Binding pair" refers to two moieties (e.g. chemical or biochemical) that have an affinity for one another. Examples of binding pairs include antigen/antibodies, lectin/avidin, target polynucleotide/probe oligonucleotide, antibody/anti-antibody, receptor/ligand, enzyme/ligand and the like. "One member of a binding pair" refers to one moiety of the pair, such as an antigen or ligand.

"Dye" refers to a molecule or part of a compound that absorbs specific frequencies of light, including but not limited to ultraviolet light. The terms "dye" and "chromophore" are synonymous.

"Fluorophore" refers to a chromophore that fluoresces.

"Membrane-permeant derivative" refers to a chemical derivative of a compound that has enhanced membrane permeability compared to an underivatized compound. Examples include ester, ether and carbamate derivatives. These derivatives are made better able to cross cell membranes, i.e. membrane permeant, because hydrophilic groups are masked to provide more hydrophobic derivatives. Also, masking groups are designed to be cleaved from a precursor (e.g., fluorogenic substrate precursor) within the cell to generate the derived substrate intracellularly. Because the substrate is more hydrophilic than the membrane permeant derivative it is now trapped within the cells.

"Isolated polynucleotide" refers to a polynucleotide of genomic, cDNA, or synthetic origin or some combination thereof, which by virtue of its origin the "isolated polynucleotide" (1) is not associated with the cell in which the "isolated polynucleotide"

is found in nature, or (2) is operably linked to a polynucleotide which it is not linked to in nature.

"Isolated protein" refers a protein, usually of cDNA, recombinant RNA, or synthetic origin or some combination thereof, which by virtue of its origin the "isolated protein" (1) is not associated with proteins that it is normally found with in nature, (2) is isolated from the cell in which it normally occurs, (3) is isolated free of other proteins from the same cellular source, e.g. free of human proteins, (4) is expressed by a cell from a different species, or (5) does not occur in nature. "Isolated naturally occurring protein" refers to a protein which by virtue of its origin the "isolated naturally occurring protein" (1) is not associated with proteins that it is normally found with in nature, or (2) is isolated from the cell in which it normally occurs or (3) is isolated free of other proteins from the same cellular source, e.g. free of human proteins.

"Polypeptide" as used herein as a generic term to refer to native protein, fragments, or analogs of a polypeptide sequence. Hence, native protein, fragments, and analogs are species of the polypeptide genus. Preferred  $G\alpha$  polypeptides, include those with the polypeptide sequence represented in the **SEQUENCE ID LISTING** and any other protein having activity similar to such  $G\alpha$  proteins as measured by one or more of the assays described herein. **SEQ. ID NO. 1** is  $G\alpha_{16}$  (murine). **SEQ. ID NO. 2** is  $G\alpha_{15}$  (human).  $G\alpha$  polypeptides or proteins can include any protein having sufficient activity for detection in the assays described herein.

"Naturally-occurring" as used herein, as applied to an object, refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control

sequences, such as when the appropriate molecules (e.g., inducers and polymerases) are bound to the control or regulatory sequence(s).

"Control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding and non-coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence; in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

"Polynucleotide" refers to a polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA.

Two amino acid sequences are homologous if there is a partial or complete identity between their sequences. For example, 85% homology means that 85% of the amino acids are identical when the two sequences are aligned for maximum matching. Gaps (in either of the two sequences being matched) are allowed in maximizing matching; gap lengths of 5 or less are preferred with 2 or less being more preferred. Alternatively and preferably, two protein sequences (or polypeptide sequences derived from them of at least 30 amino acids in length) are homologous, as this term is used herein, if they have an alignment score of at more than 5 (in standard deviation units) using the program ALIGN with the mutation data matrix and a gap penalty of 6 or greater. See Dayhoff, M.O., in Atlas of Protein Sequence and Structure, 1972, Volume 5, National Biomedical Research Foundation, pp. 101-110, and Supplement 2 to this volume, pp. 1-10. The two sequences or parts thereof are more preferably homologous if their amino acids are greater than or equal to 30% identical when optimally aligned using the ALIGN program.

"Corresponds to" refers to a sequence that is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference sequence.



The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence," "comparison window," "sequence identity," "percentage of sequence identity," and "substantial identity." A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing such as a **SEQ. ID NO. 1**, or may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 400 nucleotides in length, frequently at least 600 nucleotides in length, and often at least 800 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window," as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. (U.S.A.)* 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide

basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 50 percent sequence identity, preferably at least 60 to 70 percent sequence identity, more usually at least 80 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison.

As applied to proteins, the term "substantial identity" means that two protein sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, typically share at least 70 percent sequence identity, preferably at least 80 percent sequence identity, more preferably at least 90 percent sequence identity, and most preferably at least 95 percent sequence identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution

groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamic-aspartic, and asparagine-glutamine.

"Promiscuous Gα protein" refers to a protein with the promiscuous coupling activity of one of the Gα proteins of the SEQ. ID listing. Preferably, the promiscuous Gα protein can couple to at least one GPCR that normally couples to a Gα protein other than a promiscuous Gα protein. Examples of Gα proteins, include Gαq, Gαs, Gαi and Gα12. Promiscuous Gα protein coupling activity can be measured with an endogenously or heterologously expressed GPCR using the assays described herein. Preferably, a promiscuous Gα protein can couple to at least two different types of GPCRs that normally couple to one of the following Gα proteins, Gαq, Gαs, Gαi and Gα12. More preferably, a promiscuous Gα protein can couple to at least three different types of GPCRs that normally couple to one of the following Gα proteins, Gαq, Gαs, Gαi and Gα12. Promiscuous Gα proteins permit coupling under conditions that would not occur with a Gα protein and a receptor of a different Gα subtype, unless the Gα protein was expressed at sufficiently high levels to promote coupling with a GPCR that is not its normal coupling partner. Examples of Gα<sub>15</sub> include (Wilke, T.M. et al., PNAS, Vol. 88 pp. 10049-10053, 1991) and Gα<sub>16</sub> include (Amatruda, T.T. et al., PNAS, Vol. 88 pp. 5587-5591, 1991). It is understood that promiscuous Gα proteins do not include members of Gαq, Gαs, Gαi and Gα12 proteins that couple to only one type of GPCR

"Modulation" refers to the capacity to either enhance or inhibit a functional property of biological activity or process (e.g., enzyme activity or receptor binding); such enhancement or inhibition may be contingent on the occurrence of a specific event, such as activation of a signal transduction pathway, and/or may be manifest only in particular cell types.

The term "modulator" refers to a chemical compound (naturally occurring or non-naturally occurring), such as a biological macromolecule (e.g., nucleic acid, protein, non-peptide, or organic molecule), or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Modulators are evaluated for potential activity as inhibitors or activators (directly or indirectly) of a biological process or processes (e.g., agonist, partial antagonist, partial agonist, inverse

agonist, antagonist, antineoplastic agents, cytotoxic agents, inhibitors of neoplastic transformation or cell proliferation, cell proliferation-promoting agents, and the like) by inclusion in screening assays described herein. The activity of a modulator may be known, unknown or partially known.

"Sequence homology" refers to the proportion of base matches between two nucleic acid sequences or the proportion amino acid matches between two amino acid sequences. When sequence homology is expressed as a percentage, e.g., 50%, the percentage denotes the proportion of matches over the length of sequence from a desired sequence (e.g., SEQ. ID NO. 1) that is compared to some other sequence. Gaps (in either of the two sequences) are permitted to maximize matching; gap lengths of 15 bases or less are usually used, 6 bases or less are preferred with 2 bases or less more preferred.

The term "test chemical" refers to a chemical to be tested by one or more screening method(s) of the invention as a putative modulator.

The terms "label" or "labeled" refers to incorporation of a detectable marker, e.g., by incorporation of a radio labeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical, or colorimetric methods). Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes (e.g.,  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{35}\text{S}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (or reporter genes) (e.g., horseradish peroxidase,  $\beta$ -galactosidase,  $\beta$ -lactamase, luciferase, alkaline phosphatase), chemiluminescent, biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

"Fluorescent label" refers to incorporation of a detectable marker, e.g., by incorporation of a fluorescent moiety to a chemical entity that binds to a target or attachment to a polypeptide of biotinyl moieties that can be detected by avidin (e.g., streptavidin containing a fluorescent label or enzymatic activity that can be detected by

fluorescence detection methods). Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to dyes (e.g., FITC and rhodamine), intrinsically fluorescent proteins, and lanthanide phosphors. In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

"Reporter gene" refers to a nucleotide sequence encoding a protein that is readily detectable either by its presence or activity, including, but not limited to, luciferase, green fluorescent protein, chloramphenicol acetyl transferase,  $\beta$ -galactosidase, secreted placental alkaline phosphatase,  $\beta$ -lactamase, human growth hormone, and other secreted enzyme reporters. Generally, reporter genes encode a polypeptide not otherwise produced by the host cell, which is detectable by analysis of the cell(s), e.g., by the direct fluorometric, radioisotopic or spectrophotometric analysis of the cell(s) and preferably without the need to kill the cells for signal analysis. Preferably, the gene encodes an enzyme, which produces a change in fluorometric properties of the host cell, which is detectable by qualitative, quantitative or semi-quantitative function of transcriptional activation. Exemplary enzymes include esterases, phosphatases, proteases (tissue plasminogen activator or urokinase) and other enzymes whose function can be detected by appropriate chromogenic or fluorogenic substrates known to those skilled in the art.

"Signal transduction detection system" refers to system for detecting signal transduction across a cell membrane, typically a cell plasma membrane. Such systems typically detect at least one activity or physical property directly or indirectly associated with signal transduction. For example, an activity or physical property directly associated with signal transduction is the activity or physical property of either the receptor (e.g., GPCR), or a coupling protein (e.g., a  $G\alpha$  protein). Signal transduction detection systems for monitoring an activity or physical property directly associated with signal transduction, include GTPase activity, and conformational changes. An activity or physical property indirectly associated with signal transduction is the activity or physical property produced by a molecule other than by either the receptor (e.g., GPCR), or a coupling protein (e.g., a  $G\alpha$  protein) and associated with receptor (e.g., GPCR), or a coupling protein (e.g., a  $G\alpha$  protein). Such indirect activities and properties include

changes in intracellular levels of molecules (e.g., ions (e.g., Ca, Na or K), second messenger levels (e.g., cAMP, cGMP and inositol phosphate)), kinase activities, transcriptional activity, enzymatic activity, phospholipase activities, ion channel activities and phosphatase activities. Signal transduction detection systems for monitoring an activity or physical property indirectly associated with signal transduction, include transcriptional-based assays, enzymatic assays, intracellular ion assays and second messenger assays.

Other chemistry terms herein are used according to conventional usage in the art, as exemplified by The McGraw-Hill Dictionary of Chemical Terms (ed. Parker, S., 1985), McGraw-Hill, San Francisco, incorporated herein by reference).

#### **A DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

The invention provides cells and methods for screening or characterizing G-protein coupled receptors (GPCRs), ligands for GPCRs, and compounds that modulate signal transduction (e.g., agonists and antagonists). The term "G-protein coupled receptor" is used herein in accordance with its conventional definition. Such receptors are cell surface receptors that typically contain seven transmembrane regions and that transduce signals (e.g., sensory, hormonal, and neurotransmitter signals) from extracellular environments to intracellular environments.

Included within the invention are cells that are useful for expressing G proteins and practicing methods of the invention. A preferred cell is a stable, isolated cell that comprises a promiscuous G $\alpha$  protein construct comprising a promoter operably linked to a gene (or polynucleotide) that encodes a polypeptide with the biological activity of a promiscuous G $\alpha$  protein. A "stable isolated cell" of the invention is a cell that retains a construct typically longer than at least 3 to 4 passages in tissue culture, preferably longer than 6 to 10 passages and most preferably longer than about 12 passages. An "isolated" cell refers to a cell in an *in vitro* state (e.g., a cell of a mammalian tissue culture). The cells that are useful in the invention include both eukaryotic and prokaryotic cells that contain the constructs described herein. Preferably, the cell is a cell of a mammalian cell

line (e.g., a COS-7 cell); human cells are also preferred (e.g., a human T lymphocyte). Although not preferred, yeast cells can also be used.

A “construct,” when used in the context of molecular biology, is any genetically engineered nucleic acid (e.g., a plasmid, restriction fragment or an engineered chromosome). As used herein, a “promoter” is the minimal sequence sufficient to direct transcription of a gene (including a cDNA encoding a protein) in an eukaryote. Preferably, the promoter is derived from an eukaryotic gene or a virus that can direct transcription in an eukaryotic cell. A promoter can include a TATA box, a CAAT box, and a transcriptional start site. The term “gene” refers to a polynucleotide that encodes a protein, such as a cDNA encoding a protein.

Polypeptides that have the biological activity of a  $G\alpha$  protein are those polypeptides that are able to transduce a signal (including extracellular signals) to an effector(s) in a G-protein signaling pathway. Typically, such a polypeptide or protein, in its inactive state, is associated with GDP and the  $\beta\gamma$  dimer of a G-protein. In its “active” state, the polypeptide typically is associated with GTP and disassociated from the  $\beta\gamma$  dimer of a G-protein. The unassociated  $G\alpha$  protein is able to transduce a signal to an effector in the G-protein signaling pathway. Examples promiscuous  $G\alpha$  proteins include promiscuous  $G\alpha_{16}$  protein and a promiscuous  $G\alpha_{15}$  protein. Either promiscuous  $G\alpha_{16}$  protein or a promiscuous  $G\alpha_{15}$  protein can couple to a GPCR that normally couples to  $G_i$ ,  $G_s$  or  $G_q$  (see Fig.1). Preferably, the promiscuous  $G\alpha$  protein employed in the invention has the ability to couple with specificity to an effector in the G-protein signaling pathway. For example, the promiscuous  $G\alpha_{16}$  and  $G\alpha_{15}$  proteins each retain the ability to specifically activate the  $\beta$  isoform of phospholipase C.

Preferably, the nucleotide sequence of a promiscuous  $G\alpha$  protein has at least 70% (more preferably, at least 80% or 95%) sequence identity to the nucleotide sequence of  $G\alpha_{16}$  (SEQ ID NO: 1) and/or  $G\alpha_{15}$  (SEQ ID NO: 2). Other preferred promiscuous  $G\alpha$  proteins are those that are encoded by degenerate variants of the nucleotide sequences of promiscuous  $G\alpha_{16}$  (SEQ ID NO: 1) and/or  $G\alpha_{15}$  (SEQ ID NO: 2). A “degenerate variant” of a nucleotide sequence is a nucleotide sequence that encodes the same amino acid sequence as a given nucleotide sequence, but in which at least one codon in the nucleotide sequence is different,

because two or more different codons can encode the same amino acid. Accordingly, numerous degenerate variants can encode the promiscuous G $\alpha$  proteins of SEQ ID NO: 3 and SEQ ID NO: 4. Other preferred promiscuous G $\alpha$  protein are those that are encoded by conservative variations of the nucleotide sequences of G $\alpha_{16}$  (SEQ ID NO: 1) and/or G $\alpha_{15}$  (SEQ ID NO: 2). A “conservative variation” denotes the replacement of an amino acid residue by another, biologically similar, residue. Examples of conservative variations include the substitution of one hydrophobic residue, such as isoleucine, valine, leucine, or methionine, for another, or the substitution of one polar residue for similar polar residue, such as the substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like.

In some embodiments of the invention it will be desirable to control the level of promiscuous G $\alpha$  protein expression. High levels of promiscuous G $\alpha$  protein in a cell can deleteriously alter cell metabolism that can result in cell instability. High levels of promiscuous G $\alpha$  protein in a cell (or normal G $\alpha$  protein) can also produce high basal activities GPCRs that results in high background activities, which is not desirable for methods described herein, such as screening chemicals that may modulate receptor activity. Typically, cells having endogenously low levels of normal G $\alpha$  protein are used. Basal activity levels of GPCRs can be easily tested in a potential cell type to be used for screening with a signal transduction detection system to detect the affect of endogenously expressed G-proteins. Basal activity levels of GPCRs can also be easily tested with either endogenously expressed or heterologously expressed GPCRs in cells expressing a promiscuous G $\alpha$  protein or other G-proteins.

With GPCRs normally having high basal activity, controlled levels of promiscuous G $\alpha$  protein can help reduce background activity in a cell while achieving suitable coupling for testing putative modulators of a receptor. The amount promiscuous G $\alpha$  protein expressed in a cell can be titrated by using, or selecting for, either a weak promoter or an inducible promoter. An inducible promoter offers the advantage, compared to a weak promoter, of regulatable expression of promiscuous G $\alpha$  protein. By using an inducible promoter the amount inducer can be used to optimize the signal to



noise ratio of a screen for GPCR modulators by adjusting the amount of promiscuous Gα protein expression the cell.

An “inducible” promoter is a promoter that, in the absence of an inducer (e.g., doxycyclin) does not direct expression, or directs low levels of expression (e.g., produces less than 500 proteins per cell at steady state) of an operably linked gene (including cDNA). In the presence of an inducer, expression promiscuous Gα protein directed by the inducible promoter is typically increased at least 3-fold (preferably at least 10- 100- or 1,000-fold). Other useful inducible promoters include those that are inducible by IPTG or ecdysone. If desired, an inducible promoter can include a first promoter (e.g., a cytomegalovirus promoter) operably linked to a tet operator to regulate the first promoter (see, Gossen and Bujard, 1992, Proc. Natl. Acad. Sci. 89:5547-5551).

Many embodiments of the invention will include a polynucleotide encoding a GPCR not naturally occurring in the cell and a promiscuous Gα protein construct. The GPCR will typically not be under the control of the control sequence controlling promiscuous Gα protein expression. The GPCR may be a GPCR of known function or of protein of unknown function, such as an orphan GPCR. Promoters known in the art can be used to either constitutively or inducible express the receptor or putative receptor.

If desired, a cell of the invention can contain a polynucleotide having a control sequence and encoding a protein useful in signal transduction detection system. The construct is designed to detect activation of a Gα protein. This second construct is typically located on a second vector. It can include a reporter gene that is operably linked to a promoter that is modulated (directly or indirectly) by an active promiscuous Gα protein. Preferably, the expression of the reporter gene can be detected by detecting a change in fluorescence emission of a sample that contains the cell.

For instance, the reporter system described in PCT publication WO96/30540 (Tsien) has significant advantages over existing reporters for gene integration analysis, as it allows sensitive detection and isolation of both expressing and non-expressing single living cells. This assay system uses a non-toxic, non-polar fluorescent substrate, which is easily loaded and then trapped intracellularly. Cleavage of the fluorescent substrate by β-lactamase yields a fluorescent emission shift as substrate is converted to product.

Because the  $\beta$ -lactamase reporter readout can be ratiometric, it is unique among reporter gene assays in that it controls variables such as the amount of substrate loaded into individual cells. The stable, easily detected, intracellular readout simplifies assay procedures by eliminating the need for washing steps, which facilitates screening with cells using the invention. Preferably, a ratiometric fluorescent signal transduction detection system can be used with the invention. Preferred fluorogenic substrates are described in the Examples.

Other reporter genes such as polynucleotides encoding a polypeptide having the biological activity of green fluorescent protein (GFP) can be used.

A promoter is considered to be “modulated” by an active, promiscuous  $G\alpha$  protein when the expression of a reporter gene to which the promoter is operably linked is either increased or decreased upon activation of the promiscuous  $G\alpha$  protein. It is not necessary that the active, promiscuous  $G\alpha$  protein directly modulate reporter gene expression.

For example, embodiments of the invention presume that activation of  $G\alpha_{15}$  or  $G\alpha_{16}$  can, through a G-protein signaling pathway, activate PLC $\beta$ , which in turn increases intracellular calcium levels. An increase in calcium levels can lead to modulation of a “calcium-responsive” promoter that is part of a signal transduction detection system, i.e., a promoter that is activated (e.g., a NFAT promoter) or inhibited by a change in calcium levels. One example of an NFAT DNA binding site is found in Shaw, et al. *Science* 291:202-205 1988. Likewise, a promoter that is responsive to changes in protein kinase C levels (i.e., a “protein kinase C-responsive promoter”) can be modulated by an active  $G\alpha$  protein through G-protein signaling pathway. The cells described above can also include a G-protein coupled receptor. Genes encoding numerous GPCRs have been cloned (Simon et al., 1991, *Science* 252:802-808), and conventional molecular biology techniques can be used to express a GPCR on the surface of a cell of the invention. Preferably, the sum responsive promoter allows only a relatively short lag (e.g., less than 90 minutes) between engagement of the GPCR and transcriptional activation. A preferred responsive promoter includes the nuclear factor of activated T-cell promoter (Flanagan et al., 1991, *Nature* 352:803-807).

Many cells can be used in the invention, particularly for heterologous expression of a GPCR. Such cells include, but are not limited to; baby hamster kidney (BHK) cells (ATCC No. CCL10), mouse L cells (ATCC No. CCL1.3), Jurkats (ATCC No. TIB 152) and 153 DG44 cells [see, Chasin (1986) Cell. Molec. Genet. 12: 555] human embryonic kidney (HEK) cells (ATCC No. CRL1573), Chinese hamster ovary (CHO) cells (ATCC Nos. CRL9618, CCL61, CRL9096), PC12 cells (ATCC No. CRL17.21) and COS-7 cells (ATCC No. CRL1651). Preferred cells for heterologous cell surface protein expression are those that can be readily and efficiently transfected. Preferred cells include Jurkat cells CHO cells and HEK 293 cells, such as those described in U.S. Patent No. 5,024,939 and by Stillman et al. (1985) Mol. Cell. Biol. 5: 2051-2060.

GPCRs that can be used with the invention include, but are not limited to, muscarinic receptors, e.g., human M2 (GenBank accession #M16404); rat M3 (GenBank accession #M16407); human M4 (GenBank accession #M16405); human M5 (Bonner, et al., (1988) Neuron 1, pp. 403- 410); and the like; neuronal nicotinic acetylcholine receptors, e.g., the human  $\alpha_2$ ,  $\alpha_3$ , and  $\beta_2$ , subtypes disclosed in USSN 504,455 (filed April 3, 1990, which is hereby expressly incorporated by reference herein in its entirety); the human  $\alpha_5$  subtype (Chini, et al. (1992) Proc. Natl. Acad. Sci. U.S.A. 89: 1572-1576), the rat  $\alpha_2$  subunit (Wada, et al. (1988) Science 240, pp. 330-334); the rat  $\alpha_3$  subunit (Boulter, et al. (1986) Nature 319, pp. 368-374); the rat  $\alpha_4$  subunit (Goldman, et al. (1987) Cell 48, pp. 965-973); the rat  $\alpha_5$  subunit (Boulter, et al. (1990) I. Biol. Chem. 265, pp. 4472-4482); the chicken  $\alpha_7$  subunit (Couturier et al. (1990) Neuron 5: 847-856); the rat  $\beta_2$  subunit (Deneris, et al. (1988) Neuron 1, pp. 45-54) the rat  $\beta_3$  subunit (Deneris, et al. (1989) J. Biol. Chem. 264, pp. 6268-6272); the rat  $\beta_4$  subunit (Duvoisin, et al. (1989) Neuron 3, pp. 487-496); combinations of the rat  $\alpha$  subunits, and s  $\beta$  subunits and a and p subunits; GABA receptors, e.g., the bovine  $\alpha$ , and  $\beta_1$ , subunits (Schofield, et al. (1987) Nature 328, pp. 221-227); the bovine  $X_2$ , and  $X_3$ , subunits (Levitan, et al. (1988) Nature 335, pp. 76-79); the  $\gamma$ -subunit (Pritchett, et al. (1989) Nature 338, pp. 582-585); the  $\beta_2$ , and  $\beta_3$ , subunits (Ymer, et al. (1989) EMBO J. 8, pp. 1665-1670); the  $\delta$  subunit (Shivers, B.D. (1989) Neuron 3, pp. 327-

337); and the like; glutamate receptors, e.g., rat GluR1 receptor (Hollman, et al. (1989) Nature 342, pp. 643-648); rat GluR2 and GluR3 receptors (Boulter et al. (1990) Science 249:1033-1037; rat GluR4 receptor (Keinanen et al. (1990) Science 249: 556-560 ); rat GluR5 receptor (Bettler et al. (1990) Neuron 5: 583-595); rat GluR6 receptor (Egebjerg et al. (1991) Nature 351: 745- 748); rat GluR7 receptor (Bettler et al. (1992) neuron 8:257- 265); rat NMDAR1 receptor (Moriyoshi et al. (1991) Nature 354:31-37 and Sugihara et al. (1992) Biochem. Biophys. Res. Comm. 185:826-832); mouse NMDA el receptor (Meguro et al. (1992) Nature 357: 70-74); rat NMDAR2A, NMDAR2B and NMDAR2C receptors (Monyer et al. (1992) Science 256: 1217-1221); rat metabotropic mGluR1 receptor (Houamed et al. (1991) Science 252: 1318-1321); rat metabotropic mGluR2, mGluR3 and mGluR4 receptors (Tanabe et al. (1992) Neuron 8:169-179); rat metabotropic mGluR5 receptor (Abe et al. (1992) I. Biol. Chem. 267: 13361- 13368); and the like; adrenergic receptors, e.g., human  $\beta_1$  (Frielle, et al. (1987) Proc. Natl. Acad. Sci. 84, pp. 7920-7924); human  $\alpha_2$  (Kobilka, et al. (1987) Science 238, pp. 650-656); hamster  $\beta_2$  (Dixon, et al. (1986) Nature 321, pp. 75-79); and the like; dopamine receptors, e.g., human D2 (Stormann, et al. (1990) Molec. Pharm. 37, pp. 1-6); mammalian dopamine D2 receptor (U.S. Patent No. 5,128,254); rat (Bunzow, et al. (1988) Nature 336, pp. 783-787); and the like; and the like; serotonin receptors, e.g., human 5HT1a (Kobilka, et al. (1987) Nature 329, pp. 75-79); serotonin 5HT1C receptor (U.S. Patent No. 4,985,352); human 5HT1D (U.S. Patent No. 5,155,218); rat 5HT2 (Julius, et al. (1990) PNAS 87, pp.928-932); rat 5HT1c (Julius, et al. (1988) Science 241, pp. 558-564), and the like.

If desired (e.g., for commercial purposes), a cell(s) of the invention can be packaged into a container that is packaged within a kit. Such a kit may also contain any of the various isolated nucleic acids, antibodies, proteins, signal transduction detection systems, substrates, and/or drugs described herein, known in the art or developed in the future. A typical kit also includes a set of instructions for any or all of the methods described herein.

## METHODS OF THE INVENTION

The invention provides several methods for cloning or characterizing GPCRs, screening or characterizing ligands (e.g., known ligands) of GPCRs, and identifying or characterizing compounds that modulate signal transduction. For example, the invention provides a method for determining whether a “target” polypeptide is a GPCR for a given ligand. The method involves expressing a target polypeptide in a cell described herein that comprises a reporter gene construct (e.g., a construct encoding a  $\beta$ -lactamase reporter gene operably linked to a NFAT promoter). In this method, the test polypeptide is contacted with a chosen ligand, usually of established activity, and a change in reporter gene expression is detected. A “target” polypeptide, which is usually a GPCR, is any polypeptide expressed by a cell that can be assayed for activity using the present invention.

Similar methods can be used to test ligands and compounds using GPCRs of known, partially known and unknown function. A test ligand is a molecule that can be assayed for its ability to bind to a GPCR. A test compound is a molecule that can be assayed for its ability to modulator of signal transduction. Often, such a target polypeptide, test ligand, or test compound is, because of its sequence or structure, suspected of being able to function in a given capacity. Nonetheless, randomly chosen target polypeptides, test ligands, and test compounds also can be used in the methods described herein, and with techniques known in the art or developed in the future. For example, expression of target polypeptides from nucleic acid libraries, can be used to identify proteins involved in signal transduction, such as orphan GPCRs. For instance, this technique can be used to identify physiologically responsive receptors (e.g., taste-responsive GPCRs) where the ligand responsible for inducing a physiological event is known (e.g., a given taste sensation is known).

The invention also includes enhancement of reporter gene expression in a signal transduction detection system. This particularly useful for improving the signal to noise ratio in a screening assay. It generally involves contacting the cell with a molecule (“subthreshold regulating molecule”) that alters the activity of a cellular process to a level subthreshold to the activation of a cellularly responsive control sequence that is operably

linked to the reporter gene. Because the level of cellular activity is subthreshold, the reporter gene has a low expression level. The reporter gene system, however, is poised for activation by a change in cellular process induced by either a test chemical, test ligand or expression of target protein. Such cellularly responsive control sequences can be responsive elements known in the art in other applications. Such response elements, however, do not need be responsive to their naturally occurring signal, since the assay may occur in cells lacking the required constituents for activation by a naturally occurring signal. The subthreshold regulating molecule can either increase or decrease the activity of the cellular process. It is understood that the cellular process may not only be "classic" cellular process, such as an enzymatic activity, but it also includes levels of cellular entities (e.g., ions, metabolites and second messengers) or other measurable properties of the cell (e.g., cell volume, chromatin density, etc.). Cells described herein are preferred for this method. Other cells, however, can be used as well which express G $\alpha$  proteins endogenously, or heterologously.

For example, in order to enhance detection of expression of a reporter gene, the cell can be contacted with a compound (e.g., a calcium ionophore) that increases calcium levels inside of the cell. By increasing calcium levels inside the cell, the probability that activation of a G-protein will activate expression of a reporter gene is greatly enhanced. Preferably, the calcium levels are increased to a level that is just below the threshold level for activation of a calcium-responsive promoter, such as an NFAT promoter (see Fig. 2). In practice, ionomycin typically is added at a concentration of about 0.01 to 3  $\mu$ M, preferably 0.03 $\mu$ M. Cells described herein are preferred for this method. Other cells, however, can be used as well which express G $\alpha$  proteins endogenously, or heterologously.

In an alternate method of enhancing a signal transduction detection system, thapsigargin is added to the cell to set intracellular calcium levels at subthreshold levels to enhance reporter gene activation. Thapsigargin is added to the cell at a concentration of about 1 to 50nM, with the effect of partially depleting intracellular calcium pools and slowing the re-filling of such pools (Thastrup et al., 1990, Proc. Natl. Acad. Sci. 87:2466-2470). If desired, thapsigargin can be used at a higher concentration (e.g., 200nM to

1 $\mu$ M) in a “Ca<sup>+2</sup>-clamp” protocol, in which membrane potential is used to set the baseline calcium concentration (Negulescu et al., 1994, Proc. Natl. Acad. Sci. 91:2873-2877).

This can be applied to screening for modulators of signal transduction using a reporter gene system with a calcium-responsive promoter. Cells described herein are preferred for this method. Other cells, however, can be used as well which express G $\alpha$  proteins endogenously, or heterologously.

In yet another method of the invention, conventional molecular biology techniques can be used to express a calcium modulating ligand in cells, and thereby increase calcium levels (Bram et al., 1994, Nature 371:355-358). This can be applied to screening for modulators of signal transduction using a reporter gene system with a calcium-responsive promoter. Cells described herein are preferred for this method. Other cells, however, can be used as well which express G $\alpha$  proteins endogenously, or heterologously.

A related method of the invention for enhancing detection of expression of the reporter gene involves contacting the cell with an activator of protein kinase C. Typically, this method involves contacting the cell with 1 to 3nM of phorbol myristate acetate (PMA) or another phorbol ester; preferably PMA is used at a concentration of 0.3nM. The PMA concentration can be titrated to achieve sub threshold levels. Various analogs of PMA that retain this activity are known in the art, and can be used in the invention. Cells described herein are preferred for this method. Other cells, however, can be used as well which express G $\alpha$  proteins endogenously, or heterologously.

The invention also provides a method for determining whether a “test” ligand is a ligand for a given GPCR. In this method, a selected GPCR is expressed in a cell, such as a cell of the invention, which contains a construct and encodes a reporter gene. The cell is contacted with a test ligand, and a change in expression of the reporter gene is detected. This method is particularly well suited for identifying a ligand not known to bind to the receptor and it can also be used to determine receptor selectivity. In this method, the change in expression of the reporter gene can be compared for a sample of cells in the presence, versus in the absence, of the test ligand in order to identify ligand specific

activation. Cells described herein are preferred for this method. Other cells, however, can be used as well which express G $\alpha$  proteins endogenously, or heterologously.

The aforementioned methods can readily be adapted to provide a method for characterizing the ability of a ligand to interact with a panel of GPCRs of interest. In such an assay, the first GPCR of interest is expressed in a cell, such as a cell of the invention, that contains a construct encoding a reporter gene. In a second cell (in a second, separate sample), a second GPCR of interest is expressed along with reporter gene system. Additional GPCRs can be expressed in additional cells with reporter gene systems. Typically, these cells differ only with respect to the GPCR that is expressed. Each sample of cells is contacted with the "test" ligand of interest, and a change in reporter gene expression is detected for each cell sample. By comparing the changes in expression of the reporter gene between cell samples, one can characterize the functional activity of the ligand. This method is particularly well suited for assaying the ability of a known ligand to interact with several GPCRs that are known to be related. Thus the selectivity of the ligand can be determined. For example, various muscarinic receptors (e.g., M<sub>1</sub>, M<sub>2</sub>, and M<sub>3</sub>) can be expressed, separately, on a cell. If desired, various modulators of G-protein activity (e.g., agonists and antagonists) can be characterized in a variation of this method. Cells described herein are preferred for this method. Other cells, however, can be used as well which express G $\alpha$  proteins endogenously, or heterologously.

The invention also provides a general method for determining whether a test compound modulates signal transduction in a cell. This method also employs a cell, such as a cell of the invention, that includes a construct, and that expresses a reporter gene. In this method, the cell expresses a GPCR, and the cell is contacted with a ligand that, in the absence of a test compound, activates signal transduction. The cell is also contacted with a test compound, and a change in expression of the reporter gene indicates that the test compound modulates signal transduction in the cell.

In a variation of this method, the invention provides a "receptor-less" method for determining whether a test compound modulates signal transduction. In this variation, the cell is not engineered to express a GPCR. In lieu of contacting the cell with a ligand, the cell is



contacted with a compound that directly activates a G $\alpha$  protein encoded by a construct within the cell. Examples of such compounds include mastoparan (Calbiochem) and aluminum fluoride. These compounds typically are used at concentrations of 0.5 to 5mM. A change in expression of a reporter gene indicates that the test compound modulates signal transduction in the cell. Such a change also indicates that the compound affects signaling events that occur subsequent to receptor signaling in the signaling pathway.

The invention also provides a method for determining whether a test polypeptide is a GPCR for a given ligand, without employing a second genetic construct expressing a reporter gene. In this method, a test polypeptide is expressed in a stable, isolated cell that carries a genetic construct that includes a promoter operably linked to a gene that encodes a polypeptide having the biological activity of a promiscuous G $\alpha$  protein. The test polypeptide is contacted with a ligand, and an increase in calcium levels within the cell is detected. Any of the art-known methods for detecting a change in calcium levels can be used in this method (Negulescu and Machen, 1990, Meth. in Enzymol. 192:38-81). In a preferred method, the increase is detected by contacting the cell with fura-2 (available from Molecular Probes; Eugene, OR) and detecting a change in fluorescence emission of a sample that includes the cell.

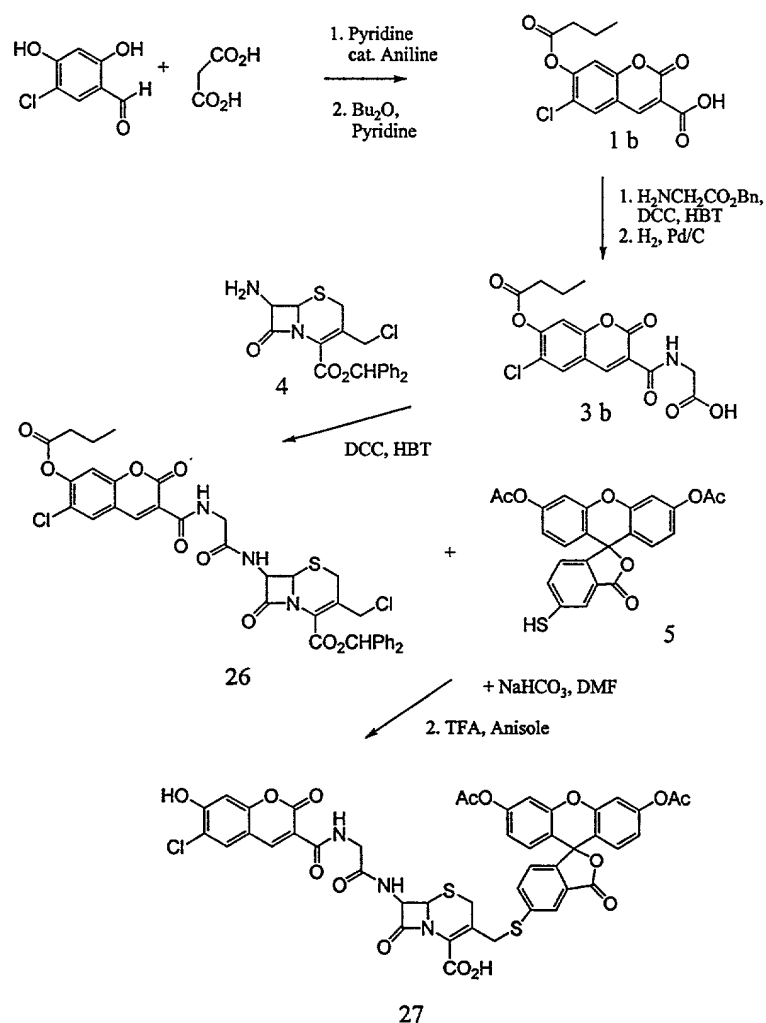
The invention offers several advantages. By employing promiscuous G-proteins, the invention allows the use of a single intracellular signaling pathway (e.g., activation of PLC $\beta$ ) to analyze GPCRs that normally couple specifically to G-proteins of a single family. By providing methods that employ living cells, the invention allows a receptor or ligand that is identified in an assay to be cloned. By employing fluorescent detection methods, the invention, in various embodiments, allows a practitioner to characterize a single cell. Accordingly, convenient cell-sorting methods, such as FACS, can be used to analyze and isolate cells. The fluorescent assays employed in the invention also provide a stable, non-labile indicator of G-protein activation. Such a stable signal (lasting up to twelve hours) allows a practitioner to analyze numerous samples in parallel, thus rendering the invention useful for high throughput screening of "test" polypeptides, ligands, and compounds. The invention provides, for the first time, an assay for associating occupancy of any GPCR with gene expression, as detected by a fluorescence



## EXAMPLES

The following examples are intended to illustrate but not limit the invention. While they are typical of those methods that might be used, other procedures known to those skilled in the art may alternatively be used.

### Example 1 - Synthesis of a $\beta$ Lactamase substrate (compound 7b)



For synthesis of 2,4 dihydroxy-5-chlorobenzaldehyde, 21.7 g (0.15 Mol) 4-chlororesorcinol were dissolved in 150 ml dry diethyl ether and 27 g finely powdered zinc (II) cyanide and 0.5 g potassium chloride were added with stirring. The suspension was cooled on ice. A strong stream of hydrogen chloride gas was blown into the solution with vigorous stirring. After approximately 30 minutes the reactants were dissolved. The addition of hydrogen chloride gas was continued until it stopped being absorbed in the ether solution (approx. 1 hour). During this time a precipitate formed. The suspension was stirred for one additional hour on ice. Then the solid was let to settle. The ethereal solution was poured from the solid. The solid was treated with 100 g of ice and heated to 100°C in a water bath. Upon cooling the product crystallized in shiny plates from the solution. They were removed by filtration and dried over potassium hydroxide. The yield was 15.9 g (0.092 Mol, 61%). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 6.23ppm (s, 1H, phenol), δ 6.62ppm (s, 1H, phenyl), δ 7.52ppm (s, 1H, phenyl), δ 9.69ppm (s, 1H, formyl), δ 11.25ppm (s, 1H, phenol).

To prepare 3-carboxy 6-chloro 7-hydroxy coumarin, 5.76 g (0.033 Mol) 2,4-dihydroxy-5-chlorobenzaldehyde and 7.2 g (0.069 Mol) malonic acid were dissolved in 5 ml warm pyridine. 75 μl Aniline were stirred into the solution and the reaction let to stand at room temperature for 3 days. The yellow solid that formed was broken into smaller pieces and 50ml ethanol was added. The creamy suspension was filtered through a glass frit and the solid was washed three times with 1 N hydrochloric acid and then with water. Then the solid was stirred with 100ml ethyl acetate, 150 ml ethanol and 10 ml half concentrated hydrochloric acid. The solvent volume was reduced in vacuo and the precipitate recovered by filtration, washed with diethyl ether and dried over phosphorous pentoxide. 4.97 g (0.021 Mol, 63%) of product was obtained as a white powder. <sup>1</sup>H NMR (dDMSO): δ 6.95ppm (s, 1H), δ 8.02ppm (s, 1H), δ 8.67ppm (s, 1H).

To prepare 7-butyryloxy-3-carboxy-6-chlorocoumarin, 3.1g (12.9 mMol) 3-carboxy-6-chloro-7-hydroxycoumarin were dissolved in 100ml dioxane and treated with 5 ml butyric anhydride, 8 ml pyridine and 20mg dimethyl aminopyridine at room temperature for two hours. The reaction solution was added with stirring to 300ml heptane upon which a white precipitate formed. It was recovered by filtration and

dissolved in 150 ml ethyl acetate. Undissolved material was removed by filtration and the filtrate extracted twice with 50 ml 1 N hydrochloric acid/brine (1:1) and then brine. The solution was dried over anhydrous sodium sulfate. Evaporation in vacuo yielded 2.63 g (8.47mMol, 66%) of product.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.08ppm (t, 3H,  $J = 7.4\text{Hz}$ , butyric methyl),  $\delta$  1.85ppm (m, 2H,  $J_1 \delta J_2 = 7.4\text{Hz}$ , butyric methylene),  $\delta$  2.68ppm (t, 2H,  $J = 7.4\text{Hz}$ , butyric methylene),  $\delta$  7.37ppm (s, 1H, coumarin),  $\delta$  7.84ppm (s, 1H, coumarin),  $\delta$  8.86ppm (s, 1H, coumarin).

Preparation of 7-butyryloxy-3-benzyloxycarbonylmethylaminocarbonyl-6-chlorocoumarin is effected as follows. 2.5g (8.06mMol) 7-Butyryloxy-3-carboxy-6-chlorocoumarin, 2.36g hydroxybenztriazole hydrate (16mMol) and 1.67g (8.1mMol) dicyclohexyl carbodiimide were dissolved in 30ml dioxane. A toluene solution of O-benzylglycine [prepared by extraction of 3.4g (10 mMol) benzylglycine tosyl salt with ethyl acetate - toluene - saturated aqueous bicarbonate - water (1:1:1:1, 250ml), drying of the organic phase with anhydrous sodium sulfate and reduction of the solvent volume to 5ml] was added drop wise to the coumarin solution. The reaction was kept at room temperature for 20 hours after which the precipitate was removed by filtration and washed extensively with ethylacetate and acetone. The combined solvent fractions were reduced to 50ml on the rotatory evaporator upon which one volume of toluene was added and the volume further reduced to 30ml. The precipitating product was recovered by filtration and dissolved in 200ml chloroform - absolute ethanol (1:1). The solution was reduced to 50ml on the rotatory evaporator and the product filtered off and dried in vacuo yielding 1.29g of the title product. Further reduction of the solvent volume yielded a second crop (0.64g). Total yield: 1.93g (4.22mMol, 52%).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.08ppm (t, 3H,  $J = 7.4\text{Hz}$ , butyric methyl),  $\delta$  1.84ppm (m, 2H,  $J_1 \delta J_2 = 7.4\text{Hz}$ , butyric methylene),  $\delta$  2.66ppm (t, 2H,  $J 7.4\text{Hz}$ , butyric methylene),  $\delta$  4.29ppm (d, 2H,  $J 5.5\text{Hz}$ , glycine methylene),  $\delta$  5.24ppm (s, 2H, benzyl),  $\delta$  7.36ppm (s, 1H, coumarin),  $\delta$  7.38ppm (s, 5H, phenyl),  $\delta$  7.77ppm (s, 1H, coumarin),  $\delta$  8.83ppm (s, 1H, coumarin),  $\delta$  9.15ppm (t, 1H,  $J 5.5\text{ Hz}$ , amide).

7-Butyryloxy-3-carboxymethylaminocarbonyl-6-chlorocoumarin was prepared as follows. 920 mg (2mMol) 7-butyryloxy-3-benzyloxycarbonylmethylamino-

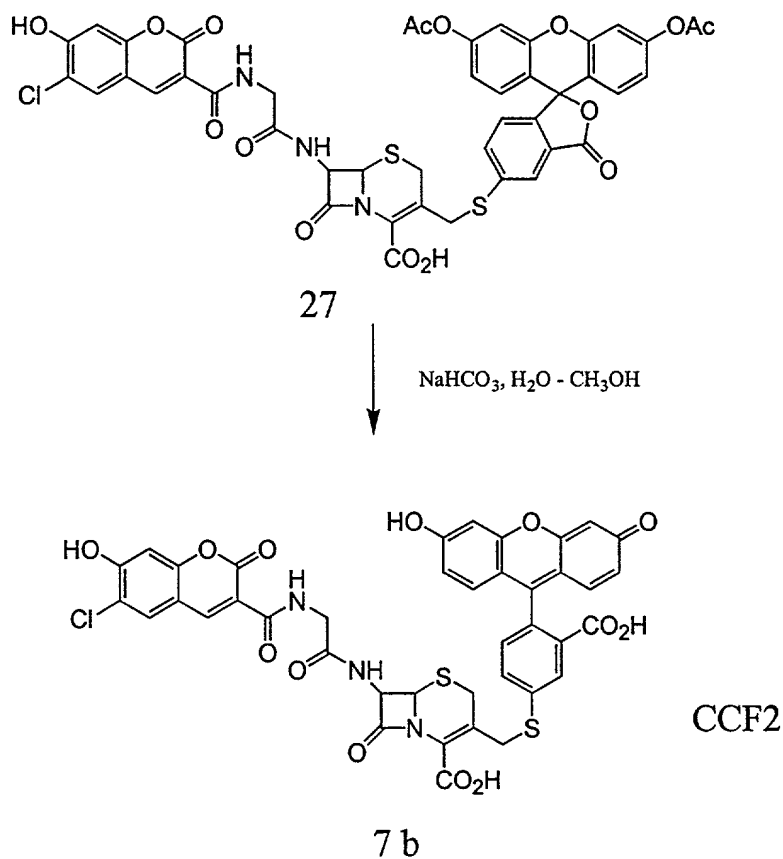
carbonyl-6-chlorocoumarin were dissolved in 50ml dioxane. 100mg Palladium on carbon (10%) and 100  $\mu$ l acetic acid were added to the solution and the suspension stirred vigorously in a hydrogen atmosphere at ambient pressure. After the uptake of hydrogen ceased the suspension was filtered. The product containing carbon was extracted five times with 25 ml boiling dioxane. The combined dioxane solutions were let to cool upon which the product precipitated as a white powder. Reduction of the solvent to 20 ml precipitates more product. The remaining dioxane solution is heated to boiling and heptane is added until the solution becomes cloudy. The weights of the dried powders were 245 mg, 389 mg and 58 mg, totaling 692 mg (1.88mMol, 94%) of white product.  $^1\text{H}$  NMR (dDMSO):  $\delta$  1.02ppm (t, 3H, J 7.4 Hz, butyric methyl),  $\delta$  1.73ppm (m, 2H,  $J_1$   $\delta$   $J_2$  = 7.3Hz, butyric methylene),  $\delta$  2.70ppm (t, 2H, J .67ppm (s, 1H, coumarin),  $\delta$  8.35ppm (s, 1H, coumarin),  $\delta$  8.90ppm (s, 1H, coumarin),  $\delta$  9.00ppm (t, 1H, J = 5.6 Hz, amide).

Coupling of 7-Butyryloxy-3-carboxymethylaminocarbonyl-6-chlorocoumarin with 7-amino-3'-chlorocephalosporanic acid benzhydryl ester was effected as follows. 368mg (1mMol) 7-Butyryloxy-3-carboxymethylaminocarbonyl-6-chlorocoumarin, 270mg hydroxybenztriazole hydrate and 415mg (1mMol) 7-amino-3'-chloro cephalosporanic acid benzhydryl ester were suspended in 40ml dioxane - acetonitrile (1:1). 260mg (1.25mMol) dicyclohexylcarbodiimide in 5ml acetonitrile were added and the suspension was stirred vigorously for 36 hours. The precipitate was removed by filtration and the volume of the solution reduced to 20ml on the rotatory evaporator. 50ml Toluene was added and the volume reduced to 30ml. With stirring 50 ml heptane was added and the suspension chilled on ice. The precipitate was recovered by filtration. It was redissolved in 10ml chloroform and the remaining undissolved solids were filtered off. Addition of 2 volumes of heptane precipitated the title product which was collected and dried in vacuo and yielded 468 mg (0.64mMol, 64%) off-white powder.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  1.08ppm (t, 3H, J 7.4Hz, butyric methyl),  $\delta$  1.84ppm (m, 2H,  $J_1$   $\delta$   $J_2$  7.4Hz, butyric methylene),  $\delta$  2.66ppm (t, 2H, J 7.4Hz, butyric methylene),  $\delta$  3.54ppm (2d, 2H, J 18.3Hz, cephalosporin C-2),  $\delta$  4.24ppm (2d, 2H, J 5.8Hz, cephalosporin 3 methylene),  $\delta$  4.37ppm (d, 2H, J 3.8Hz, glycine methylene),  $\delta$  5.02ppm (d, 1H, J 4.9Hz, cephalosporin C-6),  $\delta$  5.89ppm (dd, 1H,  $J_1$  9.0Hz,  $J_2$  5.0Hz,

cephalosporin C-7),  $\delta$  6.96ppm (s, 1H, benzhydryl),  $\delta$  7.30-7.45ppm (m, 12H, phenyl, coumarin, amide),  $\delta$  7.79ppm (s, 1H, coumarin),  $\delta$  8.84ppm (s, 1H, coumarin),  $\delta$  9.28ppm (t, 1H, J 3.7Hz, amide).

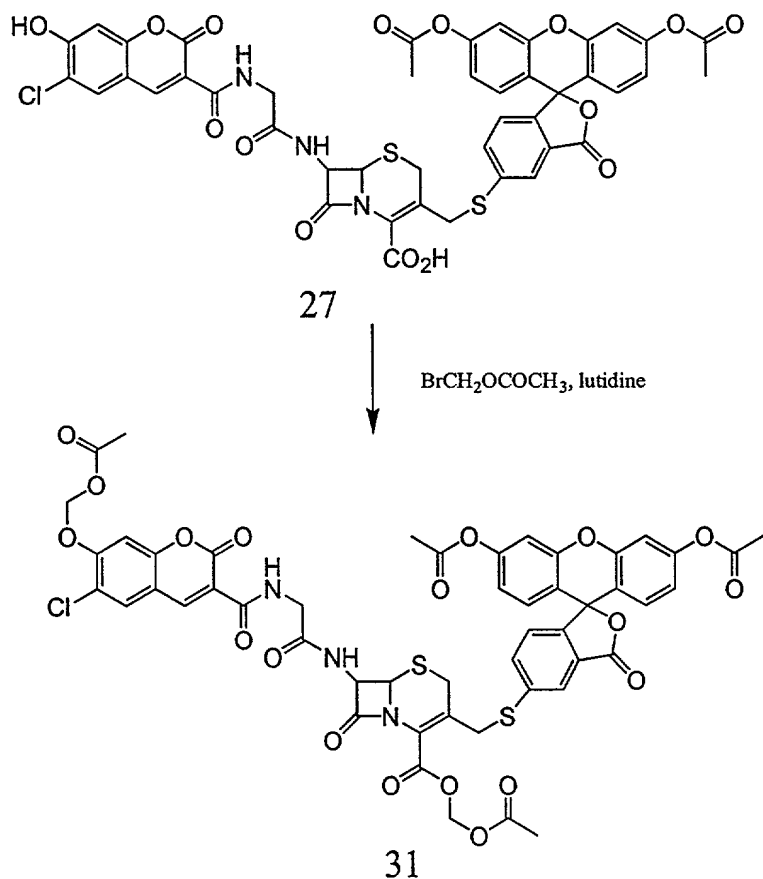
Coupling of the above product with 5-fluoresceinthiol was effected as follows. 90mg (0.2mMol) 5-mercaptofluorescein diacetate disulfide dimer were dissolved in 10ml chloroform and treated with 25  $\mu$ l tributyl phosphine and 25  $\mu$ l water in an argon atmosphere. The solution was kept for 2 hours at ambient temperature and was then added to a solution of 20mg sodium bicarbonate, 25mg sodium iodide and 110 mg (0.15mMol) of the above compound in 10ml dimethylformamide. After 4 hours the solvents were removed in vacuo and the residue triturated with diethylether. The solid was dissolved in ethyl acetate - acetonitrile (1:1). After removal of the solvents the residue was triturated once more with diethylether yielding 157mg (0.13mMol, 88%) of a cream-colored powder product.

A sample of the above compound was treated with a large excess of trifluoroacetic acid - anisole (1:1) at room temperature for 20 minutes. The reagents are removed in vacuo and the residue triturated with ether. High performance liquid chromatography of the solid in 45% aqueous acetonitrile containing 0.5% acetic acid gives a product in which the butyrate and the diphenylmethyl esters have been cleaved. It was purified by high performance liquid chromatography on a reverse phase C<sub>18</sub>-column using 45% aqueous acetonitrile containing 5% acetic acid as the eluent.



Deprotection of the fluorescein acetates in compound 27 was accomplished with sodium bicarbonate in methanol (room temperature, 30minutes) to provide the fluorescent enzyme substrate CCF2. It was purified by high performance liquid chromatography on a reverse phase C<sub>18</sub> - column using 35% aqueous acetonitrile containing 0.5% acetic acid as the eluent.





Stirring of compound 27 with excess acetoxymethyl bromide in dry lutidine produced the membrane permeable derivative of the substrate (CCF2/ac<sub>2</sub>AM<sub>2</sub>). It was purified by high performance liquid chromatography on a reverse phase C<sub>18</sub> - column using 65% aqueous acetonitrile containing 0.5% acetic acid as the eluent. CCF2/ac<sub>2</sub>AM<sub>2</sub> is readily converted to CCF2 in the cells' cytoplasm.

The donor and acceptor dyes in substrate CCF2 do not stack. The substrate is fully fluorescent in phosphate buffer and there is no formation of the "dark complex" (i.e., addition of methanol does not change the fluorescence spectrum of CCF2, except for the effect of dilution). This is due to the much smaller and more polar nature of the 7-hydroxycoumarin compared to that of the xanthene dyes (eosin, rhodamine, rhodol and resorufin).

The emission spectrum of compound CCF2 in 50 mmolar phosphate buffer pH 7.0 before and after  $\beta$ -lactamase cleavage of the  $\beta$ -lactam ring. In the intact substrate, efficient energy transfer occurs from the 7-hydroxycoumarin moiety to the

fluorescein moiety. Excitation of the substrate at 405nm results in fluorescence emission at 515nm (green) from the acceptor dye fluorescein. The energy transfer is disrupted when  $\beta$ -lactamase cleaves the  $\beta$ -lactam ring, thereby severing the link between the two dyes. Excitation of the products at 405nm now results entirely in donor fluorescence emission at 448 nm (blue). The fluorescence emission from the donor moiety increases 25 fold upon  $\beta$ -lactam cleavage. The fluorescence at 515nm is reduced by 3.5-fold, all of the remaining fluorescence originating from the 7-hydroxycoumarin as its emission spectrum extends into the green. Twenty-five-fold quenching of the donor in the substrate is equivalent to an efficiency of fluorescence energy transfer of 96%. This large fluorescence change upon  $\beta$ -lactam cleavage can readily be used to detect  $\beta$ -lactamase in the cytoplasm of living mammalian cells.

The 7-hydroxycoumarin moiety in the cephalosporin was determined to have a fluorescence quantum efficiency in the absence of the acceptor of 98-100%. This value was determined by comparing the integral of the corrected fluorescence emission spectrum of the dye with that of a solution of 9-aminoacridine hydrochloride in water matched for absorbance at the excitation wavelength. It follows that 7-hydroxycoumarin is an ideal donor dye, as virtually every photon absorbed by the dye undergoes fluorescence energy transfer to the acceptor.

### **Example 2 – Use of a $\beta$ Lactamase substrate**

Cells of the T-cell lymphoma line Jurkat were suspended in an isotonic saline solution (Hank's balanced salt solution) containing approximately  $10^{12}$   $\beta$ -lactamase enzyme molecules per milliliter (approximately 1.7nM; Penicillinase 205 TEM R<sup>+</sup>, from Sigma) and 1mg/ml rhodamine conjugated to dextran (40kd) as a marker of loading. The suspension was passed through a syringe needle (30 gauge) four times. This causes transient, survivable disruptions of the cells' plasma membrane and allows entry of labeled dextran and  $\beta$ -lactamase. Cells that had been successfully permeabilized contained  $\beta$ -lactamase and were red fluorescent when illuminated at the rhodamine excitation wavelength on a fluorescent microscope. The cells were incubated with 5 $\mu$ M fluorogenic  $\beta$ -lactamase substrate, CCF2/ac<sub>2</sub>AM<sub>2</sub>, at room temperature for 30 minutes.

Illumination with violet light (405nm) revealed blue fluorescent and green fluorescent cells. All cells that had taken up the marker rhodamine-dextran appeared fluorescent blue, while cells devoid the enzyme appeared fluorescent green.

### **Example 3 - Use of a $\beta$ Lactamase substrate**

Cells from cell lines of various mammalian origin were transiently transfected with a plasmid containing the RTEM  $\beta$ -lactamase gene under the control of a mammalian promoter. The gene encodes cytosolic  $\beta$ -lactamase lacking any signal sequence and is listed as SEQ. ID. 1. 10 to 48 hours after transfection cells were exposed to 5 micromolar CCF2/ac<sub>2</sub>AM<sub>2</sub> for 1 to 6 hours. In all cases fluorescent blue cells were detected on examination with a fluorescence microscope. Not a single blue fluorescent cell was ever detected in non transfected control cells. To quantitate the fluorescence measurements the cells were first viewed through coumarin (450 DF 65) and then fluorescein (515 EFLP) emission filters and pictures were recorded with a charge couple device camera. The average pixel intensities of CCF2 loaded transfected cells (blue) and controls (green) at coumarin and fluorescein wavelength in COS-7 (Table 2) and CHO (Table 3) cells are summarized; values for 4 representative cells for each population are given. Thus, the substrate CCF2 revealed gene expression in single living mammalian cells. Substrate can be loaded using Pluronic formulations (see Molecular Probes Catalog) using polyethylene glycol.

**Table 1**

COS-7 (origin: SV40 transformed African green monkey kidney cells)

Table of pixel intensities		coumarin emission filter	Fluorescein emission filter
Blue cell	#1	27	20
	#2	34	23
	#3	31	31
	#4	22	33
Green cell	#1	4	43
	#2	4	42
	#3	5	20
	#4	3	24

**Table 2**

CHO (origin: Chinese hamster ovary cells)

Table of pixel intensities		coumarin emission filter	Fluorescein emission filter
Blue cell	#1	98	112
	#2	70	113
	#3	76	92
	#4	56	67
Green cell	#1	9	180
	#2	9	102
	#3	7	101
	#4	9	83

**Example 4 - Expression of  $G\alpha_{15}$  and  $G\alpha_{16}$  in Cells**

This example illustrates that, although constitutive expression of  $G\alpha_{15}$  or  $G\alpha_{16}$  at high levels is toxic to cells, expression of  $G\alpha_{15}$  or  $G\alpha_{16}$  from a gene that is controlled by an inducible promoter, is tolerated by the cells. For constitutive or inducible expression of  $G\alpha_{15}$  or  $G\alpha_{16}$ , the genes encoding each of these subunits were placed, separately, under the control of a cytomegalovirus promoter in the plasmids pcDNA3G $\alpha_{15}$ , pcDNA3G $\alpha_{16}$  (Vector pcDNA3 available from Invitrogen, Inc., Del Mar, CA.), pdEF-BOSG $\alpha_{15}$ , and pdE F-BOSG $\alpha_{16}$  (For pd BOSG, see Gossen and Bujard, 1992, Proc. Natl. Acad. Sci.

89:5547-5551, also available from Clontech). To construct pdEF-BOSG 15 and pdEF-BOSG 16, sequences encoding the G subunit were inserted into pdEFBOS at its EcoRI and NotI sites. The plasmid pdEFBOS was derived from pEFBOS by removing the HindIII fragment containing the SV40 Ori (see Mizushima and Nagata, 1990, Nucl. Acids. Res. 18). Each of these plasmids was used to transfect COS-7 cells, according to conventional protocols, and each plasmid carried a *neo* gene, which confers resistance to G418. As is summarized in Table 3, approximately 150 G418-resistant clones were generated, yet none of the clones was able to express a promiscuous G-protein. The ability of a cell to express a promiscuous G-protein was determined by Western blot analysis using an antibody that binds a peptide having the amino acid sequence RPSVLARYLDEINLL (SEQ ID NO: 5) (Amatruda et al., 1991, Proc. Natl. Acad. Sci. 88:5587-5591). These data show that constitutive expression of a promiscuous G-protein under the control of a strong promoter is not tolerated by COS-7 cells. Constitutive expression of promiscuous G proteins at high levels may lead to constant accumulation of inositol phosphates or metabolites, which may be toxic to cells.

**Table 3**

High-Level Constitutive Expression of Promiscuous G-Proteins is not Tolerated

Construct	Selection	G418-resistant clones picked	Clones expressing $G\alpha_{15/16}$
PcDNA3 $G\alpha_{15}$	G418	53	0
PcDNA3 $G\alpha_{16}$	G418	48	0
pdEF-BOS $G\alpha_{15}$	G418	36	0
pdEF-BOS $G\alpha_{16}$	G418	19	0

The data summarized herein indicate that, although cells may not tolerate constitutive expression of promiscuous  $G\alpha$  proteins at high levels, they can tolerate expression of promiscuous  $G\alpha$  proteins from an inducible promoter. In this case, the genes for  $G\alpha_{15}$  and  $G\alpha_{16}$  were placed under the control of a cytomegalovirus (CMV) promoter that was operably linked to a heptamerized tet operator (Gossen and Bujard, 1992, Proc. Natl. Acad. Sci. 89:5547-5551). The plasmid encoding  $G\alpha$  and; the plasmid

encoding  $G\alpha_{16}$  are identical, except sequences encoding  $G\alpha_{16}$  in lieu of  $G\alpha_{15}$ . These plasmids were used to transfect COS-7 cells. These cells were co-transfected with a tetracyclin-dependent transactivator, rtTA, that is operably linked to a CMV promoter of a plasmid that carries a neomycin resistance gene (Gossen et al., 1995, Science 268:1766-1769).

Expression of the  $G\alpha$  genes was induced by contacting the cells with doxycyclin, a tetracyclin analog. In these experiments, the doxycyclin concentration was 3g/ml, although doxycyclin concentrations ranging from 0.01 to 10  $\mu$ /ml can be used in order to regulate the level of gene expression. Of 17 hygromycin-resistant clones that were analyzed, 2 clones showed doxycyclin-dependent expression of  $G\alpha_{15}$  or  $G\alpha_{16}$  by Western blot analysis as described above. Fig. 3 illustrates that, in the presence of doxycyclin, expression of  $G\alpha_{15}$  or  $G\alpha_{16}$  is detectable as a band of approximately 43 kDa. This expression system provides low levels of constitutive expression of  $G\alpha_{15}$  or  $G\alpha_{16}$  (e.g., less than 100  $G\alpha$  proteins/cell), yet expression of the  $G\alpha$  protein is highly inducible. Up to 10,000  $G\alpha$  proteins/cell are produced upon induction of gene expression. As a control, COS-7 cells that lacked the  $G\alpha$  gene were analyzed, and Western blot analysis indicated that the control cells did not express  $G\alpha_{15}$  or  $G\alpha_{16}$ . In sum, these experiments demonstrate that stable cells can be produced by employing an inducible promoter that provides (a) low levels of constitutive expression (i.e., producing less than approximately 100  $G\alpha$  proteins/cell), and (b) high levels of induced expression (i.e., producing approximately 10,000  $G\alpha$  proteins/cell).

#### **Example 5 - Detection of $G\alpha$ Protein Activity by Detection of Fluorescence Emission**

These examples demonstrate that activation of a  $G\alpha$  protein in a cell, and a change in expression of a reporter gene, can be detected by a detecting a change in fluorescence emission of a sample that includes the cell. These examples employ Jurkat T lymphocytes that were transfected with a genetic construct that expresses a reporter gene. The genetic construct includes a NFAT promoter, which is responsive to increased calcium levels and protein kinase C activation that result from activation of  $G\alpha$  protein. The NFAT promoter was operably linked to a  $\beta$ -lactamase reporter gene. To detect

expression of the reporter gene, and thereby detect activation of  $G\alpha$ , the cells were contacted with the  $\beta$ -lactamase substrate CCF2ac<sub>2</sub>/AM<sub>2</sub> (described herein), and fluorescence emission was detected according to previously described methods (Tsien et al., 1993, Trends in Cell Biology 3:242-245).

Two different compounds, ionomycin and phorbol myristate acetate (PMA), were used to optimize detection of expression of the reporter gene in these examples. In the first example, the dose response to ionomycin was measured. In this example, a set of samples of cells were contacted with PMA (at 3nM) and the calcium ionophore ionomycin (at various concentrations, ranging from 0 to 3.0 $\mu$ M). Ionomycin increases calcium levels inside of the cells, and thereby increases the probability that activation of a G-protein, and a G-protein-mediated increase in calcium levels, will activate expression of a reporter gene (e.g., a  $\beta$ -lactamase gene) that is operably linked to a calcium-responsive promoter (e.g., a NFAT promoter).

In practicing these methods, it is preferable to add the ionophore to a level that is just below the threshold level for activation of the calcium-responsive promoter (e.g., the NFAT promoter). Expression of the reporter gene then is activated by activation of the  $G\alpha$  protein, and the subsequent rise in intracellular calcium levels. As is illustrated in Fig.4A, fluorescence emission from a sample of the aforementioned cells can be measured by FRET. In this example, fluorescence emission was measured approximately 90 minutes after stimulation. Because the fluorogenic  $\beta$ -lactamase substrate undergoes a shift in fluorescence emission, fluorescence emission is measured as an emission ratio (450/530) when exciting at 400nm. This figure also illustrates that an ionomycin concentration of approximately 0.3 $\mu$ M is preferable for increasing the intracellular calcium level to a level that is just below the threshold level for activation of the calcium-responsive promoter.

In a second example, the dose response of PMA required to stimulate NFAT-driven expression was measured. Although PMA does not, by itself, affect NFAT-regulated gene expression, it potentiates a cell's response to an increase in calcium levels. In this example, a set of cells was treated with ionomycin (at 1 $\mu$ M ionomycin) and PMA (at various concentrations, ranging from 0 to 30nM). As above, fluorescence emission

was measured 90 minutes after stimulation. As is illustrated in Fig. 4B, increasing concentrations of PMA increased fluorescence emission from the cell sample. Thus, treating the cells with PMA enhances detection of expression of the reporter gene. This example also illustrates that a PMA concentration of approximately 3nM is preferable for enhancing detection of expression of a reporter gene.

#### **Example 6 – Monitoring Activation and Inhibition of GPCR activity with an NFAT $\beta$ -Lactamase Assay**

This example demonstrates that activation of a GPCR (Gq receptor subtype) can be detected with an NFAT  $\beta$ -lactamase assay, which is an example of signal transduction detection system based on a calcium-responsive promoter transcription based assay. Stable cell line (production of described herein) containing Gq-type GPCR receptor expresses  $\beta$ -lactamase in response to the addition of the agonist. The G $\alpha$ q protein was endogenously expressed. This response is inhibited by an antagonist. Jurkat clones expressing NFAT- $\beta$ la were transfected with expression vectors containing the Gq receptor and neomycin resistance gene (double transfection). The transfected population was neo-selected and sorted by FACS for clones responding to the GPCR agonist. For the experiments shown, cells were stimulated for three hours with the indicated ligands. Cells were then loaded with  $\beta$ -lactamase substrate CCF2/ac2AM for 1 hour, washed, dispensed into wells of a microtiter plate (100,000 cells/well) and the blue/green ratio was recorded by a plate reader.

FIG. 6 demonstrates a twenty-fold change in signal upon receptor activation with an agonist (saturating dose 100 $\mu$ M). A receptor antagonist (10 $\mu$ M) completely inhibited the agonist activation of the receptor.

#### **Example 7 – Monitoring Activation of GPCRs with a Calcium Dye (transiently transfected cells)**

This example demonstrates that activation of GPCRs (Gs and Gi receptor subtypes) can be detected with an intracellular calcium indicator transiently transfected cells, which is an example of a signal transduction detection system based on changes in



intracellular ions. The cells used were transiently transfected with two constructs. 4X 10<sup>5</sup> CHO-K1 cells were seeded on 35 mm petri dishes one day before transfection. 5 µg plasmid DNA and 12 µl lipofectamine were added for each dish using the stand method. In some cases, pBluescripts (-) or KS<sup>-</sup> plasmid was used to keep the amount of DNA consistent between each transfection. 20 h later, the cells were stained with 10 mM Fura-PE3 (Molecular Probe) for 3 h. Imaging analysis of calcium was performed to measure the [Ca<sup>2+</sup>] signal mediated by the agonists addition.

Following imaging data show that the promiscuous Gα16 couples a Gs-receptor and a Gi-receptor in CHO-K1 cells following transient transfections. The data also show that promiscuous Gα protein can change the effector downstream of the GPCR. Panel A: 60 seconds after starting of the experiment, 10 µM agonist solution was added to the cells transfected by pCIS/Gα16 (CMV promoter) and Gs-receptor (CMV promoter) expression plasmids. Panel B: 60 seconds after starting of the experiment, 10 µM agonist solution was added to the cells transfected by pCIS/Gα 16 alone. Panel C: 60 seconds after starting of the experiment, 10 µM agonist solution was added to the cells transfected by Gs receptor expression plasmid alone. Panel D: 60 seconds after starting of the experiment, 10 µM agonist solution was added to the cells transfected by pCIS/Gα16 and Gi-receptor expression plasmids. Panel E: 60 seconds after starting of the experiment, 10 µM agonist solution was added to the cells transfected by pCIS/Gα 16 alone. Panel F: 60 seconds after starting of the experiment, 10 µM agonist solution was added to the cells transfected by Gi-receptor expression plasmid alone.

### **Example 8 – Monitoring Activation of a GPCR with a Calcium Dye (stably transfected cells)**

This example demonstrates that activation of a GPCR (Gs receptor subtype) can be detected with an intracellular calcium indicator in stably transfected cells, which is an example of a signal transduction detection system based on changes in intracellular ions. The cells used were transiently stably with two constructs. Although many cells do not tolerate stable expression of promiscuous Gα protein, such as described herein, surprisingly even cells thought not to tolerate stable expression of promiscuous Gα

protein can be sorted using a signal transduction detection system. Such sorting can be performed with a high throughput sorting system, such as a FACS or 96 well imaging system. Typically, the frequency of usable stable cells is about 1 to 2 percent of those cells screened. Functional assay selection of promiscuous Gα protein/GPCR double transfected cells is a preferred method of identifying cells that either tolerate, or express the proper amounts, of promiscuous Gα protein and a GPCR.

Stable CHO-K1 cell lines expressing Gα15-Hyg alone, the Gs-receptor-Neo alone and both the Gα15 (CMV promoter) and the Gs receptor (CMV promoter) (double transfection), were generated. 48 h after transfection (described herein for the method of lipofectamine-mediated transfection), media containing Hygromycin (0.5 mg/ml), Neomycin (1 mg/ml) or both were added on to the cells to select the stable transformants. 12-15 days after selection, the stable clones were examined using the calcium imaging assays.

The following imaging data show that the promiscuous Gα15 couples a Gs-receptor in CHO-K1 cells following stable cell line generation. The data also show that promiscuous Gα protein can change the effector downstream of the GPCR.

Panel A: Calcium imaging of the Gα15/Gs-receptor dual stable clone-2. 10 μM agonist was added 40 seconds after the starting of the experiment. Panel B: Calcium imaging of the Gs-receptor stable clone -2. 10 μM agonist was added 40 seconds after the starting of the experiment. Panel C: Calcium imaging of the Gα15 stable clone-H. 10 μM agonist was added 40 seconds after the starting of the experiment.

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

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All publications, including patent documents and scientific articles, referred to in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication were individually incorporated by reference.

What is claimed is:

1. A stable, isolated cell comprising a construct with a promoter operably linked to a polynucleotide encoding a polypeptide having a biological activity of a promiscuous  $G\alpha$  protein.
2. The cell of claim 1, wherein said promoter is an inducible promoter.
3. The cell of claim 2, wherein said construct permits expression in a mammalian cells.
4. The cell of claim 1, wherein said polynucleotide has a nucleotide sequence with at least 70% sequence identity to a nucleotide sequence selected from the group consisting of the nucleotide sequence of  $G\alpha_{16}$ , SEQ ID NO: 1, the nucleotide sequence of  $G\alpha_{15}$ , and SEQ ID NO.2.
5. The cell of claim 4, wherein said polynucleotide has a nucleotide sequence selected from the group consisting of the nucleotide sequence of a  $G\alpha_{16}$  (SEQ ID NO: 1) and the nucleotide sequence of a  $G\alpha_{15}$  (SEQ ID NO:2).
6. The cell of claim 2, wherein said inducible promoter comprises a promoter operably linked to a tet operator.
7. The cell of claim 6, wherein the inducible promoter comprises a cytomegalovirus promoter.
8. The cell of claim 1, further comprising a second construct comprising a reporter gene operably linked to a second promoter, and said second promoter is modulated by a promiscuous  $G\alpha$  protein.
9. The cell of claim 8, wherein the reporter gene encodes a reporter selected from the group consisting of luciferase, green fluorescent protein, chloramphenicol acetyl transferase,  $\beta$ -galactosidase, alkaline phosphatase,  $\beta$ -lactamase, and human growth hormone.
10. The cell of claim 8, wherein said second promoter comprises a calcium-responsive promoter.
11. The cell of claim 10, wherein said calcium-responsive promoter comprises a nuclear factor of activated T cells promoter.
12. The cell of claim 11, wherein the reporter gene encodes  $\beta$ -lactamase.
13. The cell of claim 8, wherein said second promoter comprises a protein kinase C- responsive promoter.
14. The cell of claim 13, wherein the reporter gene encodes  $\beta$ -lactamase.

15. The cell of claim 1, wherein said cell further comprises a G-protein coupled receptor (GPCR).
16. The cell of claim 16, wherein said cell further comprises a polynucleotide encoding a GPCR and not naturally occurring in said cell.
17. The cell of claim 8, wherein said cell further comprises a polynucleotide encoding a GPCR and not naturally occurring in said cell.
18. An isolated cell comprising a construct with a calcium-responsive promoter operably linked to a polynucleotide encoding a reporter gene, wherein said isolated cell expresses a target protein, said calcium-responsive promoter is modulated by said target protein and with the proviso that if the function of said target protein is known, said cell is exposed to a test chemical not previously known to bind to said target protein.
19. The isolated cell of claim 18, further comprising a second polynucleotide encoding a polypeptide having a biological activity of a promiscuous G $\alpha$  protein and said second polynucleotide does not naturally occur in said cell.
20. The isolated cell of claim 19, further comprising a third polynucleotide encoding a target protein and said third polynucleotide does not naturally occur in said cell.
21. The isolated cell of claim 20, wherein said target protein has a biological activity of a GPCR.
22. The isolated cell of claim 20, wherein said target protein has a biological activity of a protein selected from the group of a transcription factor, kinase, ion channel, phosphatase, phospholipase and adenylyl cyclase.
23. The isolated cell of claim 20, further comprising a chemical known to bind to said target protein.
24. The isolated cell of claim 21, further comprising a chemical selected from the group of a phorbol ester, thapsigargin, ionomycin and a kinase inhibitor.
25. The isolated cell of claim 21, further comprising a chemical, other than a chemical in normally found in a tissue culture media, not known to bind to said target protein.
26. The isolated cell of claim 21, further comprising a cell compatible media and said chemical is at a concentration of one micromolar or less in said cell compatible media.
27. A method of identifying a GPCR for a given ligand, the method comprising:

- (i) expressing a putative GPCR in a cell of claim 1 or 18;
  - (ii) contacting said cell with a ligand; and
  - (iii) detecting reporter gene expression.
28. The method of claim 27, wherein said detecting comprises fluorescence detection.
29. The method of claim 28, further comprising contacting said cell with a reporter gene substrate.
30. The method of claim 27, further comprising contacting said cell with a compound that increases calcium levels inside said cell.
31. The method of claim 30, wherein said compound is selected from the group consisting of ionomycin and thapsigargin.
32. The method of claim 30, further comprising contacting said cell with phorbol myristate acetate or an analog thereof.
33. A method of identifying a ligand for a GPCR, the method comprising:
- a) expressing a GPCR in a cell of claim 1 or 18;
  - b) contacting said cell with a test chemical; and
  - c) detecting a signal with a signal transduction detection system.
34. The method of claim 33, further comprising comparing a signal from a first plurality of cells in the presence of said test chemical with either:
- a) a signal from a second plurality of cells in the presence of said test chemical, wherein said second plurality of cells lack either a promiscuous G $\alpha$  protein, a target protein or a reporter gene, or
  - b) a signal from a plurality of cells in the absence of said test chemical, wherein said plurality of cells are substantially the same as said first plurality of cells.
35. The method of claim 34, wherein said detecting comprises fluorescence detection.
36. The method of claim 35, wherein said cell comprises a dye.
37. The method of claim 36, wherein said detecting comprises reporter gene detection.

38. A method for identifying a modulator of signal transduction in a cell, the method comprising:
- a) contacting a cell of claim 1 or 18 with a test chemical;
  - b) contacting said cell with a ligand that, in the absence of the test chemical, activates signal transduction in said cell, and
  - c) detecting a signal with a signal transduction detection system
39. A method for identifying modulators of signal transduction in a cell, the method comprising:
- a) contacting a cell of claim 1 or 18 with a compound that directly activates a G subunit encoded by a polynucleotide,
  - b) contacting said cell with a test chemical, and
  - c) detecting a signal with a signal transduction detection system, wherein said signal transduction detection system comprises a reporter gene as a measure of signal transduction in said cell.
40. The method of claim 39, wherein said compound is selected from the group consisting of mastoparan and aluminum fluoride.
41. A method for identifying a GPCR for a given ligand or method of identifying a modulator of a GPCR, the method comprising:
- a) expressing a putative GPCR or a GPCR of known function in a cell of claim 1 or 18;
  - b) contacting said cell with a test chemical or a ligand known to be a GPCR; and
  - c) detecting a calcium level within said cell.
42. The method of claim 41, further comprising contacting said cell with intracellular calcium indicator and detecting fluorescence from said cell.
43. A kit comprising assay reagents and a container containing a cell of claim 1 or 18.
44. A stable, isolated cell comprising a construct with a promoter operably linked to a polynucleotide encoding a polypeptide having a biological activity of a promiscuous G $\alpha$  protein.
45. The cell of claim 44, wherein said promoter is a CMV promoter.
46. The cell of claim 44, wherein said construct permits expression in a mammalian cells.
47. The cell of claim 46, wherein said polynucleotide has a nucleotide sequence with at least 70% sequence identity to a nucleotide sequence selected from the group consisting of the

nucleotide sequence of  $G\alpha_{16}$ , SEQ ID NO: 1, the nucleotide sequence of  $G\alpha_{15}$ , and SEQ ID NO.2.

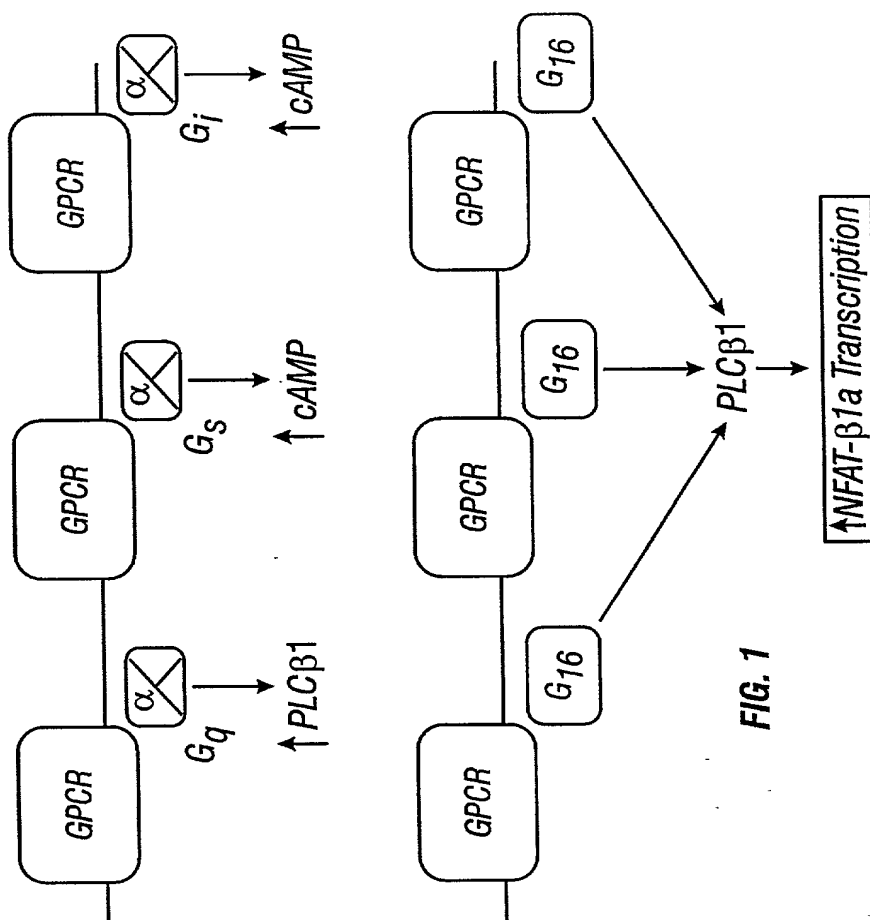
48. The cell of claim 47, wherein said polynucleotide has a nucleotide sequence selected from the group consisting of the nucleotide sequence of a  $G\alpha_{16}$  (SEQ ID NO: 1) and the nucleotide sequence of a  $G\alpha_{15}$  (SEQ ID NO:2).
49. The cell of claim 44, further comprising a second construct comprising a reporter gene operably linked to a second promoter, and said second promoter is modulated by a promiscuous  $G\alpha$  protein.
50. The cell of claim 49, wherein the reporter gene encodes a reporter selected from the group consisting of luciferase, green fluorescent protein, chloramphenicol acetyl transferase,  $\beta$ -galactosidase, alkaline phosphatase,  $\beta$ -lactamase, and human growth hormone.
51. The cell of claim 50, wherein said second promoter comprises a calcium-responsive promoter.
52. The cell of claim 51, wherein said calcium-responsive promoter comprises a nuclear factor of activated T cells promoter.
53. The cell of claim 52, wherein the reporter gene encodes  $\beta$ -lactamase.
54. The cell of claim 51, wherein said second promoter comprises a protein kinase C- responsive promoter.
55. The cell of claim 54, wherein the reporter gene encodes  $\beta$ -lactamase.
56. The cell of claim 44, wherein said cell further comprises a G-protein coupled receptor (GPCR).
57. The cell of claim 56, wherein said cell further comprises a polynucleotide encoding a GPCR and not naturally occurring in said cell.
58. The cell of claim 54, wherein said cell further comprises a polynucleotide encoding a GPCR and not naturally occurring in said cell.
59. The cell of claim 58, wherein said cell arises from a cell line subjected to functional cells analysis with a signal transduction detection system.
60. A method of identifying a ligand for a GPCR, the method comprising:



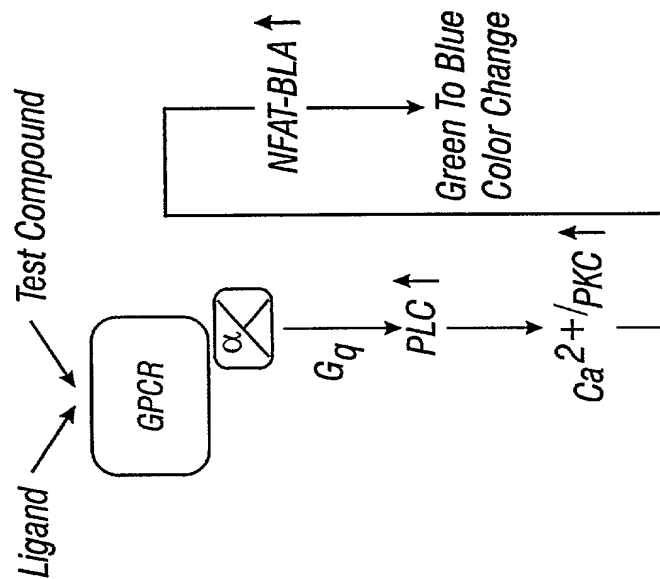
- a) contacting a cell with a test chemical, wherein said cell is expressing a GPCR and arises from a cell line subjected to functional cell analysis with a signal transduction detection system; and
  - b) detecting a signal with a signal transduction detection system.
61. A method for identifying modulators of signal transduction in a cell, the method comprising:
- a) contacting a cell with a compound that directly activates a  $G\alpha$  protein encoded by a polynucleotide, wherein said cell arises from a cell line subjected to functional cell analysis with a signal transduction detection system,
  - b) contacting said cell with a test chemical, and
  - c) detecting a signal with a signal transduction detection system, wherein said signal transduction detection system comprises a reporter gene as a measure of signal transduction in said cell.
62. A method for identifying a GPCR for a given ligand or method of identifying a modulator of a GPCR, the method comprising:
- a) expressing a putative GPCR or a GPCR of known function in a cell, wherein said cell arises from a cell line subjected to functional cell analysis with a signal transduction detection system;
  - b) contacting said cell with a test chemical or a ligand known to be a GPCR; and
  - c) detecting a calcium level within said cell.

## Abstract

Disclosed are compositions and methods for their use, such as in identifying G-protein coupled receptors and ligands and compounds that modulate signal transduction. The compositions and methods employ promiscuous G-proteins. Activation of the promiscuous G-protein can be detected in a variety of assays, including assays in which activation is indicated by a change in fluorescence emission of a sample that contains the composition.



**FIG. 1**



**FIG. 2**

*Hygro*

S

18

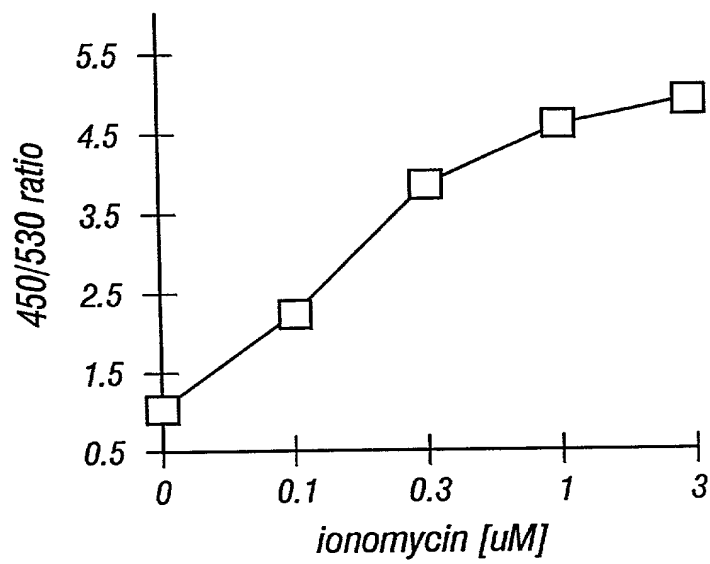
12

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*Doxycylin*  
(3ug/ml)

*kDa*

**FIG. 3**



**FIG. 4A**

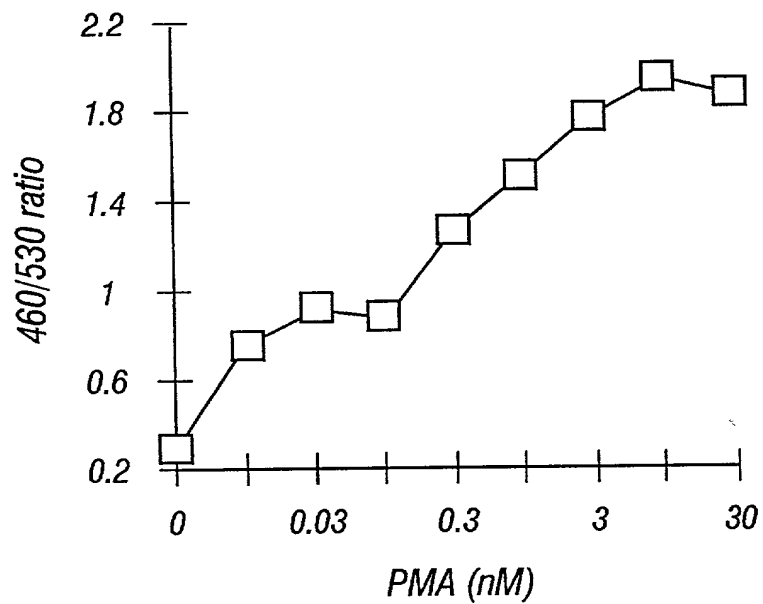


FIG. 4B

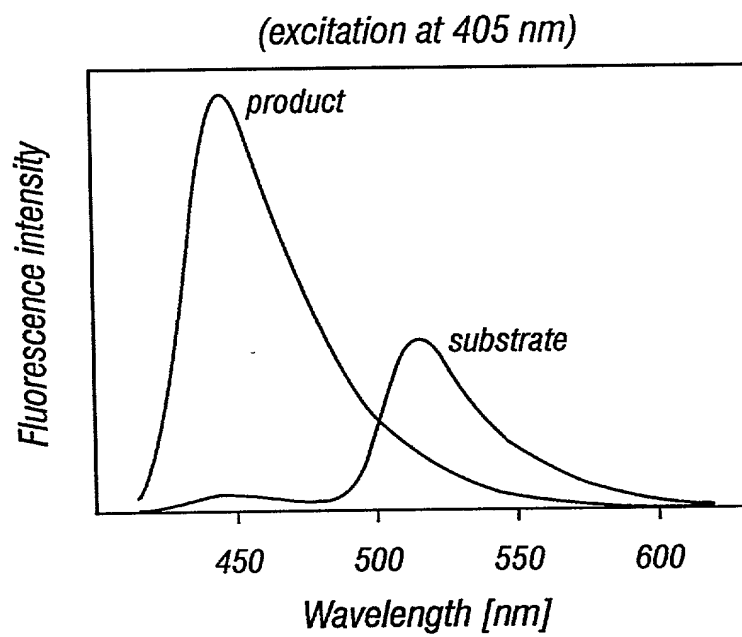


FIG. 5

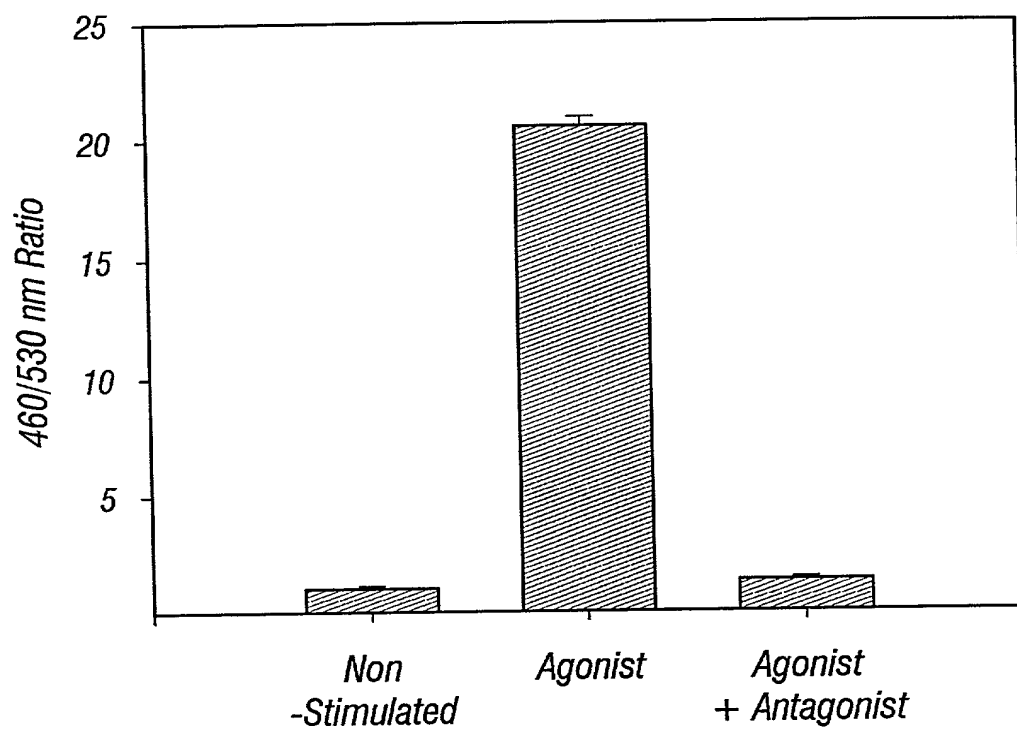


FIG. 6

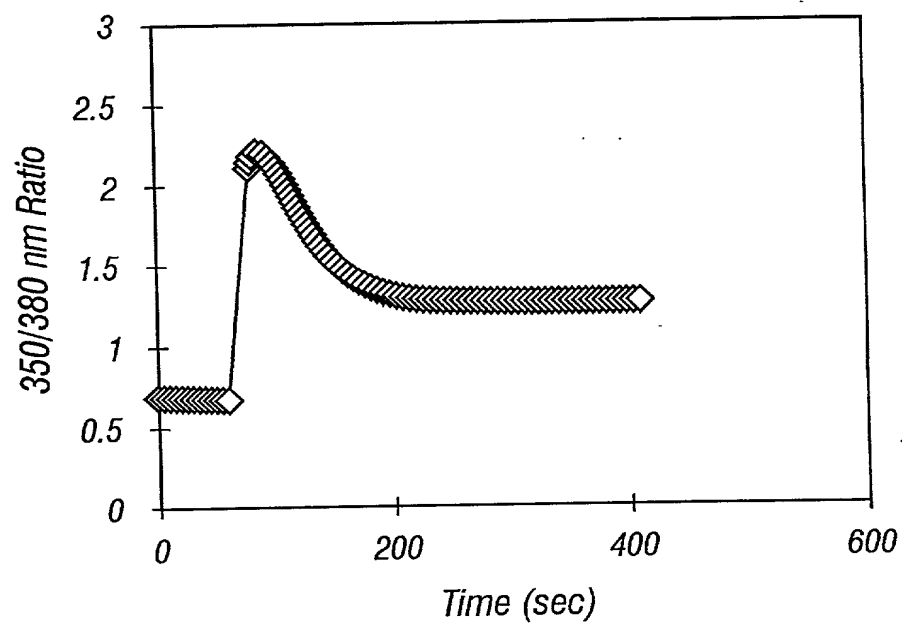


FIG. 7A

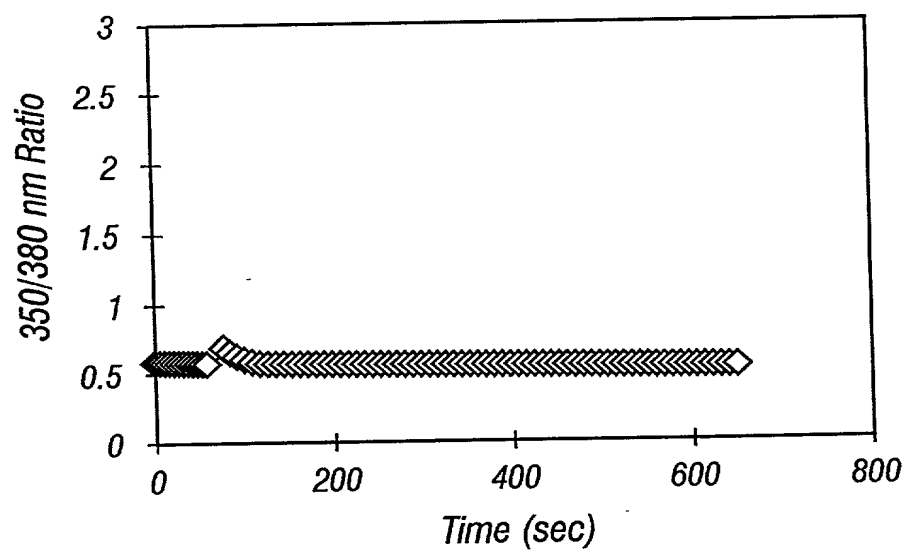


FIG. 7B

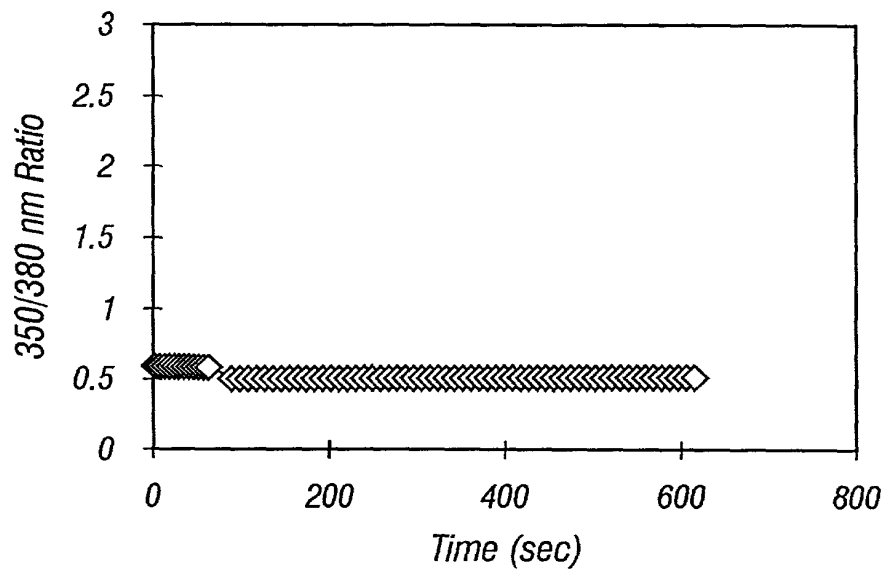


FIG. 7C

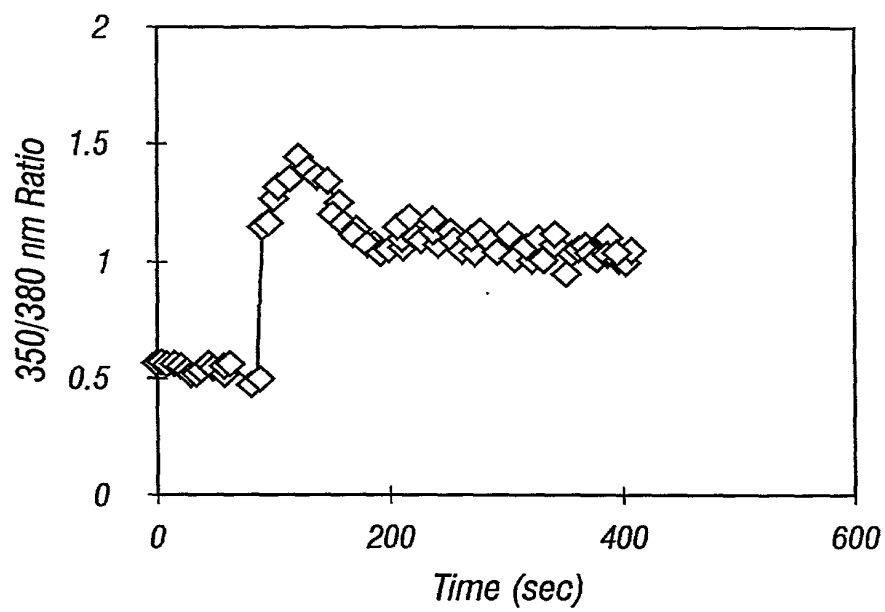


FIG. 7D



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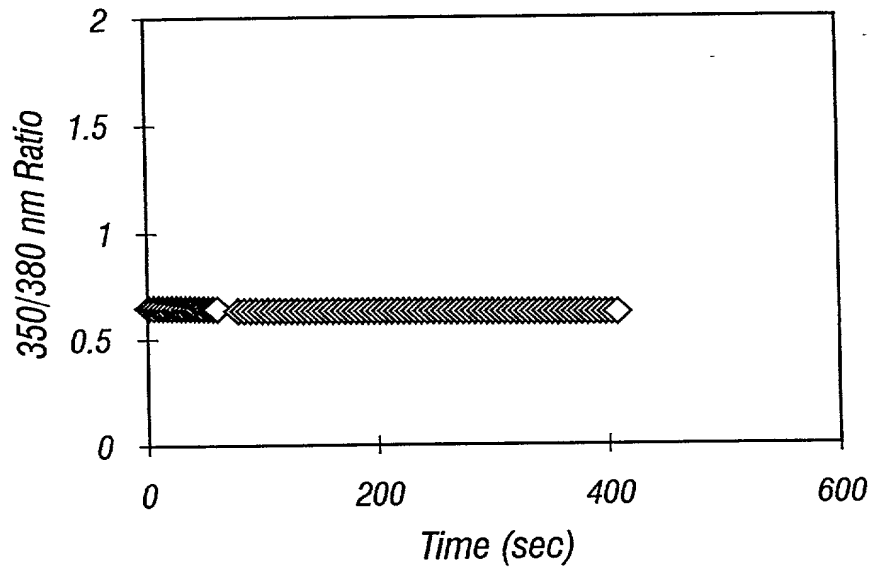


FIG. 7E

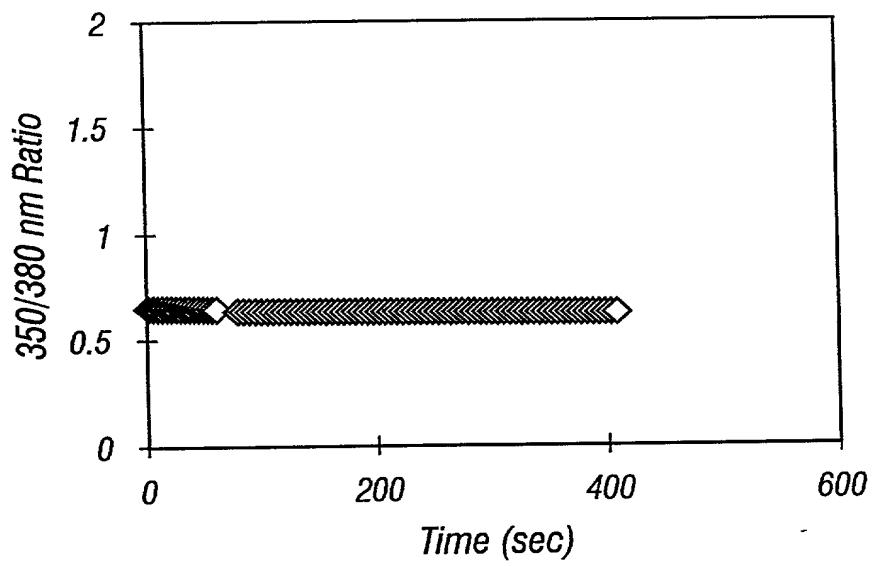


FIG. 7F

FIG. 8A

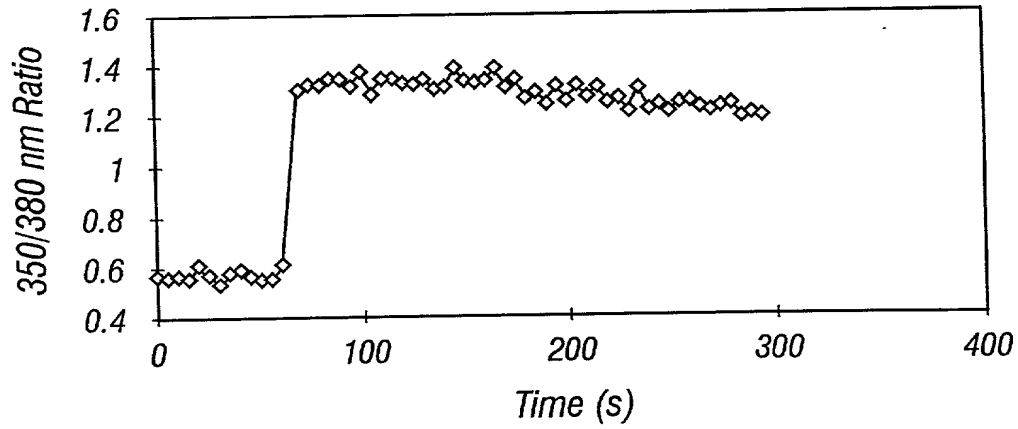


FIG. 8B

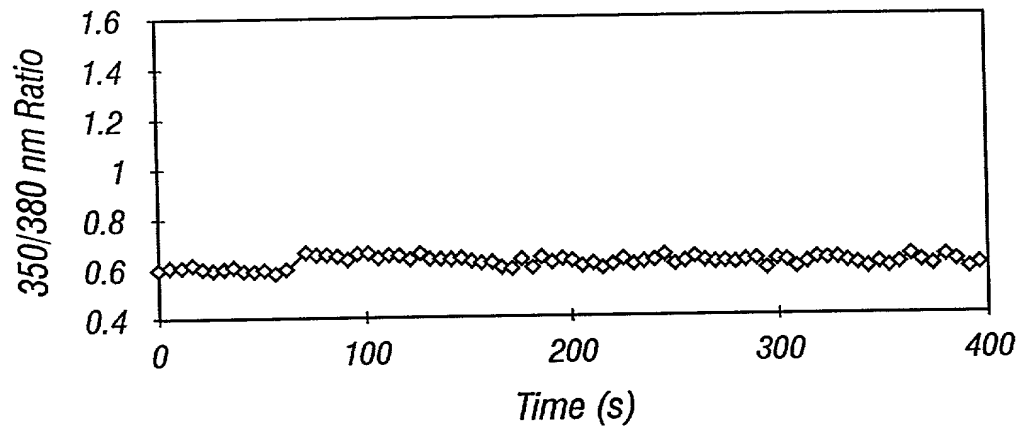
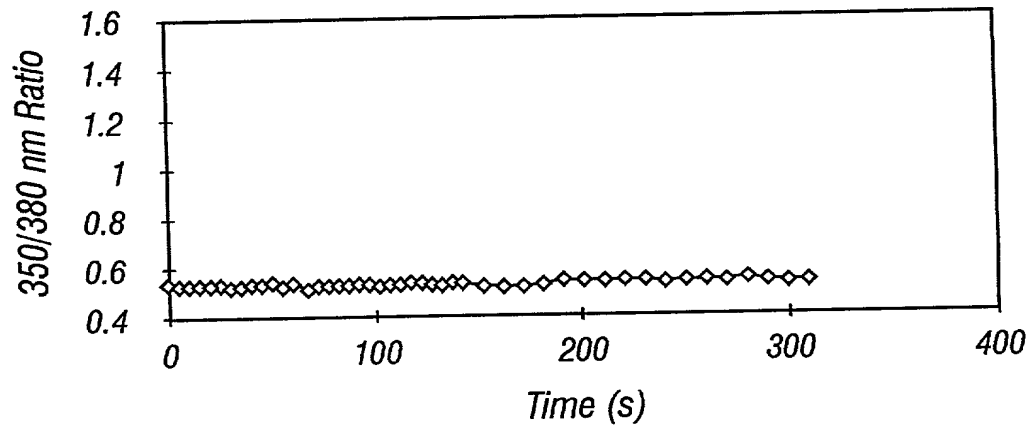


FIG. 8C



SUBSTITUTE COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled PROMISCUOUS G-PROTEINS COMPOSITIONS AND THEIR USE, the specification of which

☐ is attached hereto.

☒ was filed on 06/19/97 as Application Serial No. 08/878,801  
and was amended on \_\_\_\_\_.

☐ was described and claimed in PCT International Application No. \_\_\_\_\_  
filed on \_\_\_\_\_ and as amended under  
PCT Article 19 on \_\_\_\_\_.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim the benefit under Title 35, United States Code, §119(e)(1) of any United States provisional application(s) listed below:

U.S. SERIAL NO.	FILING DATE	STATUS
<u>60/020,234</u>	<u>6/21/96</u>	<u>EXPIRED</u>

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Lisa A. Haile, Reg. No. 38,347; Richard J. Imbra, Reg. No. 37,643; June M. Learn, Reg. No. 31,238; Terrance A. Meador, Reg. No. 30,298; Stephen E. Reiter, Reg. No. 31,192, of Gray Cary Ware & Freidenrich LLP.

Address all telephone calls to Lisa A. Haile at telephone number 858/677-1456.

Address all correspondence to Lisa A. Haile, Gray Cary Ware & Freidenrich LLP, 4365 Executive Drive, Suite 1600, San Diego, CA 92121-2189

PATENT

ATTORNEY DOCKET NO: 08366/002001

SUBSTITUTE COMBINED DECLARATION AND POWER OF ATTORNEY CONTINUED

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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Date:

Residence Address:

Berlin, Germany

Citizen of:

Germany

Post Office Address:

Johannisberger Str. 27, 14197, Berlin, Germany

Full Name of Inventor:

Melvin Simon

Inventor's Signature:

Date:

Residence Address:

1075 Old Mill Road, San Marino, CA 91108

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Post Office Address:

1075 Old Mill Road, San Marino, CA 91108

Full Name of Inventor:

Charles Zuker

Inventor's Signature:

Date:

Residence Address:

4778 Thurston Place, San Diego, CA 92130

Citizen of:

Post Office Address:

4778 Thurston Place, San Diego, CA 92130

## SUBSTITUTE COMBINED DECLARATION AND POWER OF ATTORNEY CONTINUED

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Full Name of Inventor: Paul Negulescu

Inventor's Signature: \_\_\_\_\_ Date: \_\_\_\_\_

Residence Address: 678 Solana Circle, Solana Beach, CA 92075

Citizen of: \_\_\_\_\_

Post Office Address: 678 Solana Circle, Solana Beach, CA 92075Full Name of Inventor: Stefan OffermannsInventor's Signature: Stefan Offermanns Date: 9/6/99Residence Address: Berlin, GermanyCitizen of: GermanyPost Office Address: Johannistberger Str. 27, 14197, Berlin, GermanyFull Name of Inventor: Melvin Simon

Inventor's Signature: \_\_\_\_\_ Date: \_\_\_\_\_

Residence Address: 1075 Old Mill Road, San Marino, CA 91108

Citizen of: \_\_\_\_\_

Post Office Address: 1075 Old Mill Road, San Marino, CA 91108Full Name of Inventor: Charles Zuker

Inventor's Signature: \_\_\_\_\_ Date: \_\_\_\_\_

Residence Address: 4778 Thurston Place, San Diego, CA 92130

Citizen of: \_\_\_\_\_

Post Office Address: 4778 Thurston Place, San Diego, CA 92130

Revised: August 24 1994 (391DECL.MRG)

PATENT  
ATTORNEY DOCKET NO: 08366/002001

SUBSTITUTE COMBINED DECLARATION AND POWER OF ATTORNEY CONTINUED

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Full Name of Inventor: Paul Negulescu  
Inventor's Signature: \_\_\_\_\_ Date: \_\_\_\_\_  
Residence Address: 678 Solana Circle, Solana Beach, CA 92075  
Citizen of: \_\_\_\_\_  
Post Office Address: 678 Solana Circle, Solana Beach, CA 92075

Full Name of Inventor: Stefan Offermanns  
Inventor's Signature: \_\_\_\_\_ Date: \_\_\_\_\_  
Residence Address: Berlin, Germany  
Citizen of: Germany  
Post Office Address: Johannisberger Str. 27, 14197, Berlin, Germany

Full Name of Inventor: Melvin Simon  
Inventor's Signature: Melvin Simon Date: 9/9/99  
Residence Address: 1075 Old Mill Road, San Marino, CA 91108  
Citizen of: U.S.  
Post Office Address: 1075 Old Mill Road, San Marino, CA 91108

Full Name of Inventor: Charles Zuker  
Inventor's Signature: \_\_\_\_\_ Date: \_\_\_\_\_  
Residence Address: 4778 Thurston Place, San Diego, CA 92130  
Citizen of: \_\_\_\_\_  
Post Office Address: 4778 Thurston Place, San Diego, CA 92130

**PATENT**  
**ATTORNEY DOCKET NO: 08366/002001**

**SUBSTITUTE COMBINED DECLARATION AND POWER OF ATTORNEY CONTINUED**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Full Name of Inventor: Paul Negulescu

Inventor's Signature: \_\_\_\_\_ Date: \_\_\_\_\_

Residence Address: 678 Solana Circle, Solana Beach, CA 92075

Citizen of: \_\_\_\_\_

Post Office Address: 678 Solana Circle, Solana Beach, CA 92075

Full Name of Inventor: Stefan Offermanns

Inventor's Signature: \_\_\_\_\_ Date: \_\_\_\_\_

Residence Address: Berlin, Germany

Citizen of: Germany

Post Office Address: Johannisberger Str. 27, 14197, Berlin, Germany

Full Name of Inventor: Melvin Simon

Inventor's Signature: \_\_\_\_\_ Date: \_\_\_\_\_

Residence Address: 1075 Old Mill Road, San Marino, CA 91108

Citizen of: \_\_\_\_\_

Post Office Address: 1075 Old Mill Road, San Marino, CA 91108

Full Name of Inventor: Charles Zuker

Inventor's Signature:  Date: 9/24/99

Residence Address: 4778 Thurston Place, San Diego, CA 92130

Citizen of: USA

Post Office Address: 4778 Thurston Place, San Diego, CA 92130

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Negulescu et al.

Parent Application No.: 08/878,801

Parent Filed: June 19, 1997

Application No.: Unassigned

Filed: Herewith

For: AMIDASE

) Group Art Unit: Unassigned

)

) Examiner: Unassigned

)

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

"EXPRESS MAIL" MAILING LABEL NO.: EL382208175US

DATE OF DEPOSIT 12/20/99 I HEREBY CERTIFY THAT THIS  
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Mikhail Bayley  
(TYPED OR PRINTED NAME OF PERSON MAILING PAPER OR FEE)

Mikhail Bayley  
(SIGNATURE OF PERSON MAILING PAPER OR FEE)

Assistant Commissioner for Patents  
Washington, D.C. 20231

**PERMISSION TO USE SEQUENCE LISTING**

Sir:

The above-identified patent application lacks a substitute paper copy of the Sequence Listing for inclusion into the Specification, as well as a computer readable form of the Sequence Listing. Applicants respectfully direct the attention of the Office to the following:

1. A complete paper copy of the Sequence Listing is to be inserted following the Abstract on page 60 and before the drawings. Please delete pages 46 through 53 of the Sequence Listing and insert the Sequence Listing beginning with page 1 and numbering consecutively thereafter. This paper copy is identical to the paper copy of the Sequence Listing which is included in the Specification of U.S. Patent Application Serial No. 08/878,801 (the "801 application").



2. A computer-readable form of the Sequence Listing identical to the paper copy of the Sequence Listing is on file in the '801 application. Pursuant to 37 CFR §1.821(e), reference to the computer-readable form on file in the '801 application is hereby made, which computer-readable form was filed in the '801 application on August 5, 1998. Said computer-readable form is identical to both the paper copy of the Sequence Listing on file in the '801 application and the paper copy of the Sequence Listing submitted in the present application.

Applicants submit that the foregoing satisfies the requirements of Rule §1.821. If there are any questions regarding this response, the Office is invited to contact the undersigned.

The Assistant Commissioner is hereby authorized to charge payment of the following fees associated with this communication or credit any overpayment to Deposit Account No. 07-1895. A duplicate copy of this sheet is enclosed.

Respectfully submitted,

Dated: 12/20/99



Lisa A. Haile, Ph.D.  
Registration No. 38,347  
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San Diego, CA 92121-2189

# SEQUENCE LISTING

<110> Negulescu, Paul  
Offermanns, Stefan  
Simon, Melvin  
Zuker, Charles

<120> PROMISCUOUS G-PROTEINS COMPOSITIONS AND THEIR USE

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<140> US 08/878,801

<141> 1997-06-19

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## SEQUENCE ID LISTING

### Nucleotide and Amino Acid Sequences of Gα15

(SEQ ID NO: 2 and SEQ ID NO: 4, respectively)

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ATG	GCC	CGG	TCC	CTG	ACT	TGG	GGC	TGC	TGT	CCC	TGG	TGC	CTG	ACA	GAG	GAG	GAG
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Met	Ala	Arg	Ser	Leu	Thr	Trp	Gly	Cys	Cys	Pro	Trp	Cys	Leu	Thr	Glu	Glu	Glu
63	72	81	90	99	108												
AAG	ACT	GCC	GCC	AGA	ATC	GAC	CAG	GAG	ATC	AAC	AGG	ATT	TTG	TTG	GAA	CAG	AAA
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Lys	Thr	Ala	Ala	Arg	Ile	Asp	Gln	Glu	Ile	Asn	Arg	Ile	Leu	Leu	Glu	Gln	Lys
117	126	135	144	153	162												
AAA	CAA	GAG	CGC	GAG	GAA	TTG	AAA	CTC	CTG	CTG	TTG	GGG	CCT	GGT	GAG	AGC	GGG
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Lys	Gln	Glu	Arg	Glu	Glu	Leu	Lys	Leu	Leu	Leu	Leu	Gly	Pro	Gly	Glu	Ser	Gly
171	180	189	198	207	216												
AAG	AGT	ACG	TTC	ATC	AAG	CAG	ATG	CGC	ATC	ATT	CAC	GGT	GTG	GGC	TAC	TCG	GAG
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Lys	Ser	Thr	Phe	Ile	Lys	Gln	Met	Arg	Ile	Ile	His	Gly	Val	Gly	Tyr	Ser	Glu
225	234	243	252	261	270												
GAG	GAC	CGC	AGA	GCC	TTC	CGG	CTG	CTC	ATC	TAC	CAG	AAC	ATC	TTC	GTC	TCC	ATG
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Glu	Asp	Arg	Arg	Ala	Phe	Arg	Leu	Leu	Ile	Tyr	Gln	Asn	Ile	Phe	Val	Ser	Met



279	288	297	306	315	324
CAG GCC ATG ATA GAT GCG ATG GAC CGG CTG CAG ATC CCC TTC AGC AGG CCT GAC					
---	---	---	---	---	---
Gln Ala Met Ile Asp Ala Met Asp Arg Leu Gln Ile Pro Phe Ser Arg Pro Asp					
333	342	351	360	369	378
AGC AAG CAG CAC GCC AGC CTA GTG ATG ACC CAG GAC CCC TAT AAA GTG AGC ACA					
---	---	---	---	---	---
Ser Lys Gln His Ala Ser Leu Val Met Thr Gln Asp Pro Tyr Lys Val Ser Thr					
387	396	405	414	423	432
TTC GAG AAG CCA TAT GCA GTG GCC ATG CAG TAC CTG TGG CGG GAC GCG GGC ATC					
---	---	---	---	---	---
Phe Glu Lys Pro Tyr Ala Val Ala Met Gln Tyr Leu Trp Arg Asp Ala Gly Ile					
441	450	459	468	477	486
CGT GCA TGC TAC GAG CGA AGG CGT GAA TTC CAC CTT CTG GAC TCC GCG GTG TAT					
---	---	---	---	---	---
Arg Ala Cys Tyr Glu Arg Arg Arg Glu Phe His Leu Leu Asp Ser Ala Val Tyr					
495	504	513	522	531	540
TAC CTG TCA CAC CTG GAG CGC ATA TCA GAG GAC AGC TAC ATC CCC ACT GCG CAA					
---	---	---	---	---	---
Tyr Leu Ser His Leu Glu Arg Ile Ser Glu Asp Ser Tyr Ile Pro Thr Ala Gln					
549	558	567	576	585	594
GAC GTG CTG CGC AGT CGC ATG CCC ACC ACA GGC ATC AAT GAG TAC TGC TTC TCC					
---	---	---	---	---	---
Asp Val Leu Arg Ser Arg Met Pro Thr Thr Gly Ile Asn Glu Tyr Cys Phe Ser					
603	612	621	630	639	648
GTG AAG AAA ACC AAA CTG CGC ATC GTG GAT GTT GGT GGC CAG AGG TCA GAG CGT					
---	---	---	---	---	---
Val Lys Lys Thr Lys Leu Arg Ile Val Asp Val Gly Gly Gln Arg Ser Glu Arg					

657	666	675	684	693	702
AGG AAA TGG ATT CAC TGT TTC GAG AAC GTG ATT GCC CTC ATC TAC CTG GCC TCC					
----	----	----	----	----	----
Arg Lys Trp Ile His Cys Phe Glu Asn Val Ile Ala Leu Ile Tyr Leu Ala Ser					
711	720	729	738	747	756
CTG AGC GAG TAT GAC CAG TGC CTA GAG GAG AAC GAT CAG GAG AAC CGC ATG GAG					
----	----	----	----	----	----
Leu Ser Glu Tyr Asp Gln Cys Leu Glu Glu Asn Asp Gln Glu Asn Arg Met Glu					
765	774	783	792	801	810
GAG AGT CTC GCT CTG TTC AGC ACG ATC CTA GAG CTG CCC TGG TTC AAG AGC ACC					
----	----	----	----	----	----
Glu Ser Leu Ala Leu Phe Ser Thr Ile Leu Glu Leu Pro Trp Phe Lys Ser Thr					
819	828	837	846	855	864
TCG GTC ATC CTC TTC CTC AAC AAG ACG GAC ATC CTG GAA GAT AAG ATT CAC ACC					
----	----	----	----	----	----
Ser Val Ile Leu Phe Leu Asn Lys Thr Asp Ile Leu Glu Asp Lys Ile His Thr					
873	882	891	900	909	918
TCC CAC CTG GCC ACA TAC TTC CCC AGC TTC CAG GGA CCC CGG CGA GAC GCA GAG					
----	----	----	----	----	----
Ser His Leu Ala Thr Tyr Phe Pro Ser Phe Gln Gly Pro Arg Arg Asp Ala Glu					
927	936	945	954	963	972
GCC GCC AAG AGC TTC ATC TTG GAC ATG TAT GCG CGC GTG TAC GCG AGC TGC GCA					
----	----	----	----	----	----
Ala Ala Lys Ser Phe Ile Leu Asp Met Tyr Ala Arg Val Tyr Ala Ser Cys Ala					
981	990	999	1008	1017	1026
GAG CCC CAG GAC GGT GGC AGG AAA GGC TCC CGC GCG CGC CGC TTC TTC GCA CAC					
----	----	----	----	----	----
Glu Pro Gln Asp Gly Gly Arg Lys Gly Ser Arg Ala Arg Arg Phe Phe Ala His					

1035	1044	1053	1062	1071	1080
TTC ACC TGT GCC ACG GAC ACG CAA AGC GTC CGC AGC GTG TTC AAG GAC GTG CGG					
----	----	----	----	----	----
Phe Thr Cys Ala Thr Asp Thr Gln Ser Val Arg Ser Val Phe Lys Asp Val Arg					

1089	1098	1107	1116	1125
GAC TCG GTG CTG GCC CGG TAC CTG GAC GAG ATC AAC CTG CTG TGA				
----	----	----	----	----
Asp Ser Val Leu Ala Arg Tyr Leu Asp Glu Ile Asn Leu Leu ***				

Nucleotide and Amino Acid Sequences of G 16  
(SEQ ID NO: 1 and SEQ ID NO: 3, respectively)

9	18	27	36	45	54
ATG GCC CGC TCG CTG ACC TGG CGC TGC TGC CCC TGG TGC CTG ACG GAG GAT GAG					
----	----	----	----	----	----
Met Ala Arg Ser Leu Thr Trp Arg Cys Cys Pro Trp Cys Leu Thr Glu Asp Glu					

63	72	81	90	99	108
AAG GCC GCC GCC CGG GTG GAC CAG GAG ATC AAC AGG ATC CTC TTG GAG CAG AAG					
----	----	----	----	----	----
Lys Ala Ala Ala Arg Val Asp Gln Glu Ile Asn Arg Ile Leu Leu Glu Gln Lys					

117	126	135	144	153	162
AAG CAG GAC CGC GGG GAG CTG AAG CTG CTG CTT TTG GGC CCA GGC GAG AGC GGG					
----	----	----	----	----	----
Lys Gln Asp Arg Gly Glu Leu Lys Leu Leu Leu Leu Gly Pro Gly Glu Ser Gly					

171	180	189	198	207	216
AAG AGC ACC TTC ATC AAG CAG ATG CGG ATC ATC CAC GGC GCC GGC TAC TCG GAG					
----	----	----	----	----	----
Lys Ser Thr Phe Ile Lys Gln Met Arg Ile Ile His Gly Ala Gly Tyr Ser Glu					

225	234	243	252	261	270
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GAG GAG CGC AAG GGC TTC CGG CCC CTG GTC TAC CAG AAC ATC TTC GTG TCC ATG  
 ---  
 Glu Glu Arg Lys Gly Phe Arg Pro Leu Val Tyr Gln Asn Ile Phe Val Ser Met  
  
 279 288 297 306 315 324  
 CGG GCC ATG ATC GAG GCC ATG GAG CGG CTG CAG ATT CCA TTC AGC AGG CCC GAG  
 ---  
 Arg Ala Met Ile Glu Ala Met Glu Arg Leu Gln Ile Pro Phe Ser Arg Pro Glu  
  
 333 342 351 360 369 378  
 AGC AAG CAC CAC GCT AGC CTG GTC ATG AGC CAG GAC CCC TAT AAA GTG ACC ACG  
 ---  
 Ser Lys His His Ala Ser Leu Val Met Ser Gln Asp Pro Tyr Lys Val Thr Thr  
  
 387 396 405 414 423 432  
 TTT GAG AAG CGC TAC GCT GCG GCC ATG CAG TGG CTG TGG AGG GAT GCC GGC ATC  
 ---  
 Phe Glu Lys Arg Tyr Ala Ala Ala Met Gln Trp Leu Trp Arg Asp Ala Gly Ile  
  
 441 450 459 468 477 486  
 CGG GCC TGC TAT GAG CGT CGG CGG GAA TTC CAC CTG CTC GAT TCA GCC GTG TAC  
 ---  
 Arg Ala Cys Tyr Glu Arg Arg Arg Glu Phe His Leu Leu Asp Ser Ala Val Tyr  
  
 495 504 513 522 531 540  
 TAC CTG TCC CAC CTG GAG CGC ATC ACC GAG GAG GGC TAC GTC CCC ACA GCT CAG  
 ---  
 Tyr Leu Ser His Leu Glu Arg Ile Thr Glu Glu Gly Tyr Val Pro Thr Ala Gln  
  
 549 558 567 576 585 594  
 GAC GTG CTC CGC AGC CGC ATG CCC ACC ACT GGC ATC AAC GAG TAC TGC TTC TCC  
 ---  
 Asp Val Leu Arg Ser Arg Met Pro Thr Thr Gly Ile Asn Glu Tyr Cys Phe Ser  
  
 603 612 621 630 639 648  
 GTG CAG AAA ACC AAC CTG CGG ATC GTG GAC GTC GGG GGC CAG AAG TCA GAG CGT

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--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Val Gln Lys Thr Asn Leu Arg Ile Val Asp Val Gly Gly Gln Lys Ser Glu Arg

      657      666      675      684      693      702
AAG AAA TGG ATC CAT TGT TTC GAG AAC GTG ATC GCC CTC ATC TAC CTG GCC TCA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Lys Lys Trp Ile His Cys Phe Glu Asn Val Ile Ala Leu Ile Tyr Leu Ala Ser

      711      720      729      738      747      756
CTG AGT GAA TAC GAC CAG TGC CTG GAG GAG AAC AAC CAG GAG AAC CGC ATG AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Leu Ser Glu Tyr Asp Gln Cys Leu Glu Glu Asn Asn Gln Glu Asn Arg Met Lys

      765      774      783      792      801      810
GAG AGC CTC GCA TTG TTT GGG ACT ATC CTG GAA CTA CCC TGG TTC AAA AGC ACA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Glu Ser Leu Ala Leu Phe Gly Thr Ile Leu Glu Leu Pro Trp Phe Lys Ser Thr

      819      828      837      846      855      864
TCC GTC ATC CTC TTT CTC AAC AAA ACC GAC ATC CTG GAG GAG AAA ATC CCC ACC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ser Val Ile Leu Phe Leu Asn Lys Thr Asp Ile Leu Glu Glu Lys Ile Pro Thr

      873      882      891      900      909      918
TCC CAC CTG GCT ACC TAT TTC CCC AGT TTC CAG GGC CCT AAG CAG GAT GCT GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ser His Leu Ala Thr Tyr Phe Pro Ser Phe Gln Gly Pro Lys Gln Asp Ala Glu

      927      936      945      954      963      972
GCA GCC AAG AGG TTC ATC CTG GAC ATG TAC ACG AGG ATG TAC ACC GGG TGC GTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ala Ala Lys Arg Phe Ile Leu Asp Met Tyr Thr Arg Met Tyr Thr Gly Cys Val

      981      990      999      1008      1017      1026
GAC GGC CCC GAG GGC AGC AAG AAG GGC GCA CGA TCC CGA CGC CTT TTC AGC CAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

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Asp Gly Pro Glu Gly Ser Lys Lys Gly Ala Arg Ser Arg Arg Leu Phe Ser His

1035	1044	1053	1062	1071	1080
TAC	ACA	TGT	GCC	ACA	GAC
ACA	CAG	AAC	ATC	CGC	AAG
GTC	TTC	AAG	GAC	GTG	CGG
---	---	---	---	---	---
Tyr	Thr	Cys	Ala	Thr	Asp
Thr	Gln	Asn	Ile	Arg	Lys
Val	Phe	Lys	Asp	Val	Arg

1089	1098	1107	1116	1125										
GAC	TCG	GTG	CTC	GCC	CGC	TAC	CTG	GAC	GAG	ATC	AAC	CTG	CTG	TGA
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Asp	Ser	Val	Leu	Ala	Arg	Tyr	Leu	Asp	Glu	Ile	Asn	Leu	Leu	***